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Keynote Symposium (1)

1

Cell Biology in the Genomic Era.

F. Collins; National Human Genome Research Institute/NIH, Bethesda, MD

The Human Genome Project completed all of its original goals in 2003, including an essentially complete sequence of the human genome and a variety of other tools for its analysis. Since that time, an expanding set of ambitious production projects have been contributing substantial new insights to genome function. Among those projects are: 1) a comparative genomic approach to the sequencing of dozens of vertebrates and invertebrates; 2) development of a dense catalogue of human genetic variation; 3) the establishment of a collection of full length cDNA clones; 4) a project to knock out every single one of the mouse genes; 5) a comprehensive approach to cancer known as The Cancer Genome Atlas; 6) an ambitious Roadmap effort to characterize the genomes of the microbes that live on and in the human body (The Human Microbiome Project); and 7) a Roadmap initiative to provide small molecule screening capabilities for academic investigators. Perhaps the most relevant of these projects for cell biology, however, are the ENCODE and modENCODE projects, These aim to identify the functional components of the human, fly, and worm genomes, bringing together dozens of laboratories to work together using cutting edge genome technologies, with the commitment to provide all of this information immediately over the internet. After a successful pilot effort devoted to studying 1% of the human genome, the ENCODE project has now scaled up to tackle the entire human DNA instruction book, and is rapidly providing information including transcript maps, binding sites for modified histones, DNase hypersensitive sites, promoter locations, and transcription factor binding sites across the genome. Researchers in cell biology should find their efforts to answer fundamental biological questions considerably empowered by the expanding availability of these and other public databases of information about genome function.

Symposium I: Cell Biology of the Senses (2-4)

2

The Mechanotransduction Machinery of Hair Cells.

U. Mueller; Department of Cell Biology, The Scripps Research Institute, La Jolla, CA

Mechanosensation, the transduction of mechanical force into an electrochemical signal, allows living organisms to detect touch, hear, register movement and gravity, and sense changes in cell volume and shape. Hair cells in the vertebrate inner ear are the specialized mechanoreceptor cells for the detection of sound and head movement. Each hair cell contains at the apical surface rows of stereocilia that are connected by extracellular filaments into an exquisitely organized bundle. Mechanotransduction channels are localized towards the tips of stereocilia and gated by an elastic element, the gating spring, which is stretched upon stereocilia deflection and mediates rapid channel opening. Channel opening is followed by an adaptation process, which keeps the channels in their most sensitive range of operation. Components of the mechanotransduction machinery in hair cells have recently been identified by the analysis of genes that are linked to deafness. These studies indicate that some forms of hearing loss can be classified as a new class of diseases that is directly caused by defects in the mechanotransduction process.

3

Scensational Cell Biology: Trafficking and Function of Insect Odorant Receptors.

L. B. Vosshall[1,2]; [1]Laboratory of Neurogenetics and Behavior, The Rockefeller University, New York, NY, [2]Howard Hughes Medical Institute, New York, NY

Insects are both beneficial and deleterious to human health and happiness. While honeybees are the principal pollinators of our crops, mosquitoes are major vectors of human infectious diseases and a wide range of insect pests damage human agricultural products. Olfaction—the sense of smell—is an important sensory modality used by insects to find plants and animals to feed on. Recent findings suggest that insects have adopted an evolutionarily distinct family of membrane proteins to detect odor cues. These seven transmembrane domain proteins adopt a topology distinct from the more conventional seven transmembrane domain G protein-coupled receptors that vertebrates and nematodes use to sense odors. Moreover, the insect chemosensory receptors are heteromultimeric protein complexes composed of a variable ligand binding subunit and a constant subunit called Or83b that is critical for targeting the receptor to the ciliated dendrites of olfactory sensory neurons. There is emerging evidence that these atypical receptors function as odor-gated ion channels, but the extent to which these receptors depend on G protein signaling for their function remains a topic of intense debate in the field. This symposium presentation will take a comparative approach to discuss our current understanding of the biogenesis, assembly, trafficking and function of insect chemosensory receptors as contrasted with the same processes in the vertebrate nose.

4

TRP Channels in Sensory Signaling and Neurodegenerative Disease.

C. Montell; Department of Biological Chemistry, Johns Hopkins University, Baltimore, MD

TRP channels endow us with the ability to sense tastants, mechanical stimuli, a variety of chemicals, distinct temperatures and in some animals, light and pheromones. The founding member of the TRP family functions in fly phototransduction, downstream of a rhodopsin/Gq/phospholipase C signaling cascade. Some of the molecules that function in this cascade, such as arrestin, undergo light dependent translocation between different subcellular compartments, and we have shown that this dynamic shuttling requires a phototransduction cascade distinct from the classical Gq/PLC pathway. During the last few years it has become clear that TRP channels are global and evolutionarily conserved mediators of sensory signaling in all animals. Our recent studies demonstrate that the exquisite sensitivity of Drosophila larvae to small deviations from their ideal temperature of 18 °C occurs by indirect activation of a TRP channel downstream of a phototransduction-like cascade, rather than by direct activation of a thermoTRP. We propose that indirect activation of a TRP channel via a signaling cascade promotes amplification of small differences in temperature and facilitates adaptation to temperatures within the comfortable range. Finally, mutations in TRP channels underlie many human diseases, including the neurodegenerative disease, MLIV. Our recent studies clarify the cellular mechanism underlying this disease, and provide the conceptual framework for developing strategies for treating this debilitating early childhood disorder.

Bruce Alberts Award for Excellence in Science Education (5)

5

Science Education for Civic Engagement.

K. K. Oates[1], W. Burns[2]; [1]DUE, NSF, Arlington, VA, [2]National Center Science and Civic Engagement, Harrisburg University, Harrisburg, PA

The recipients of the 2008 Bruce Alberts Award will describe Science Education for New Civic Engagements and Responsibilities (SENCER), the work for which they are being recognized. SENCER applies the science of learning to the learning of science, all to expand civic capacity. An NSF-supported faculty development and dissemination program, SENCER improves science education by linking the discovery of scientific principles and the development of scientific thinking to engagement with complex social problems. By developing faculty expertise in teaching "to" basic science and mathematics "through" civic concerns, the SENCER approach extends the impact of learning across the curriculum and into the community. SENCER courses and programs provide ready and convincing answers to a common question college students ask: "what do I need this for?" The original SENCER course focused on the "multidisciplinary trouble" of HIV disease as a strategy to teach basic biology and introduce students to the complex set of trans-disciplinary and public policy matters attending to the pandemic. Today, the SENCER program features 37 model courses and programs on a wide range of issues from sustainability to

sleep. Using materials, assessment instruments, and research developed in the SENCER project, faculty design and implement curricular projects that (1) get more students interested and engaged in learning in STEM courses, (2) help students connect STEM learning to their other studies, and (3) strengthen students' understanding of science and their capacity for responsible work and citizenship. Having involved more than 1300 academic leaders from 330 colleges and universities from 47 states and 13 foreign nations, the SENCER program has established a community of scholars engaged in improving educational practice. This vibrant community is sustained through five regional centers for innovation, an intensive Summer Institute for faculty development, annual Capitol Hill Symposia in which students and faculty present their work, a digital library, an on line peer-reviewed journal, and an extensive set of assessment resources. The SENCER web site is: www.sencer.net.

Symposium II: Chromatin Organization and Gene Expression (6-8)

6

The Rapid and Locus-Wide Transition of a Gene from a Poised to a Highly Activated State.

J. T. Lis[1], K. Kieckhafer[1], S. Petesch[1], W. W. Webb[2], J. Yao[2], K. Zobeck[2]; [1]Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY, [2]School of Applied and Engineering Physics, Cornell University, Ithaca, NY

Hsp70 loci/genes experience dramatic changes in their chromatin and transcription factor association during the seconds and minutes following heat shock gene activation. We are using multiphoton microscopy to examine the dynamics of GFP- and photoactivatable GFP-tagged Pol II and specific transcription factors at particular chromosomal loci in real time and in living cells. We are also using biochemical mapping methods to examine the kinetics and location of the changes in chromatin structure that occur at heat shock loci in response to gene activation. Interestingly, the dramatic loss in nucleosomes at activated heat shock loci precedes the movement of Pol II through the gene. Moreover, the region affected covers a domain that is larger than the transcription unit, and extends to, but not beyond, the known insulator regions that flank the Hsp70 genes. This induced change is dependent upon the upstream activators, HSF and GAGA factor. Additionally, we have found that Poly(ADP)-Ribose Polymerase and its enzymatic activity are critical for this dramatic and rapid change in chromatin architecture and are investigating the underlying mechanisms.

7

When Structure Meets Function: Organizing the Yeast Nucleus.

S. Gasser[1,3], A. Taddei[2], E. Van Nimwegen[3], H. Schober[1], G. Van Houwe[2], H. Ferreira[1]; [1]Friedrich Miescher Institute for Biomedical Research, Basel, Switzerland, [2]Institut Curie, Paris, France, [3]University of Basel, Basel, Switzerland

We study how the higher-order organization of chromatin in chromosomes and interphase nuclei affects genome function. Chromosomes are organized into domains of active and inactive chromatin, often called eu- and heterochromatin. We have examined heterochromatic domains in yeast by characterizing the biochemical structure of the transcriptionally silent, SIR-complexed nucleosomal fiber, and its subnuclear positioning. Yeast silent chromatin clusters in foci at the nuclear perimeter, held in place by the interaction of a structural protein of silent chromatin with Esc1, which is associated with the nuclear envelope but which is excluded from pores. A second anchoring pathway requires yKu and the membrane spanning protein Mps3. In contrast, active galactose- and stress-induced genes are shown to accumulate at pores. Both the inter-pore positioning of silent domains and the pore-association of active genes impact activity: heritable repression for the former, and optimal mRNA expression of the latter. We next examined the importance of such perinuclear subcompartments for regulating the transcription of genes located far from telomeres. Microarray analyses were performed under conditions that eliminated telomere anchoring, yet maintained SIR factor integrity. Among the genes whose expression increased upon SIR factor release, many were within 20 kb of a telomere, confirming that NE anchoring helps regulate natural subtelomeric genes. The loci that were down-regulated upon loss of telomere anchoring were distributed over all chromosomes. Half of this ectopic repression was SIR-complex dependent. A global analysis that correlates changes in gene expression with transcription factor binding sites revealed that promoters bearing the PAC motif or binding sites for Abf1, were consistently down-regulated by SIR release. We conclude that SIR sequestration in perinuclear clusters not only promotes subtelomeric repression, but prevents promiscuous silencing at a subset of promoters.

8

The Spatial Organization of Genomes.

T. Misteli; National Cancer Institute/NIH, Bethesda, MD

A recently discovered fundamental hallmark of genomes is their non-random spatial organization in the cell nucleus. Entire chromosomes and single genes occupy preferential positions within the nucleus of higher eukaryotes. These positions are cell type- and tissue-specific and they change during physiological processes such as differentiation, development and in disease. The higher order non-random arrangement of chromosomes and genes has now been implicated in all major nuclear processes including gene regulation, DNA replication, and DNA repair. We find that spatial genome organization plays a particularly important role in maintenance of genome stability and formation of chromosomal translocations. Mapping the spatial position of chromosomes and gene loci in the interphase nucleus indicates that the non-random organization of the genome contributes to the formation of chromosomal translocations. To further test the role of spatial genome dynamics in genome maintenance we have developed experimental systems to visualize DNA double strand breaks and DNA repair factors in living cells. Results form these approaches have implications for our conceptual and mechanistic understanding of how cancer translocations form, and how DNA damage is prevented and detected in vivo.

E. E. Just Lecture (9)

9

Host Cell Cholesterol Homeostasis and HIV Replication.

H. Taylor[1], I. C. Leao[2], Y. Tang[1], R. Broughton[1], J. E. Hildreth[1]; [1]Center for AIDS Health Disparities Research, Meharry Medical College, School of Medicine, Nashville, TN, [2]Cancer Immunology, The Johns Hopkins University School of Medicine, Baltimore, MD

HIV-1 replication is critically dependent on host cell cholesterol homeostasis. The ability of various inhibitors of cholesterol biosynthesis to abrogate HIV-1 replication underscores the significance of cholesterol in HIV biology. Previous studies by our laboratory and others indicate that cholesterol-rich regions of cell membranes are essential for HIV-1 entry and egress. These studies firmly establish the important structural role that cholesterol plays in virus-host cell membrane fusion and budding events. Recent studies demonstrate that HIV-1 infection results in alterations of host cell cholesterol homeostasis via inhibition of cholesterol efflux and coordinated expression of cholesterol transport and biosynthetic genes. HIV-1 infection activates sterol-responsive element binding protein 2 (SREBP-2), which orchestrates the synchronized transcription of genes in the cholesterol biosynthesis pathway. This up-regulation is mediated via sterol regulatory elements (SRE) present in the promoters of these genes. In our current studies we have identified the multifunctional transcription factor TFII-I as a novel SREBP-responsive gene. In earlier studies TFII-I was found to play a critical role in HIV-1 LTR transcription. Using a combination of biochemical, pharmacological and genetic approaches we demonstrate for the first time how TFII-I links cholesterol metabolism to HIV transcription. In other studies, we examine the role of the SREBP-target gene NPC-1 in HIV replication. NPC-1 is associated with a rare cholesterol-storage disorder, Niemann Pick Type C disease, characterized by defects in cholesterol trafficking in cells. We show that NPC-1 genetic deficiency or chemical inhibition of the NPC pathway results in a reduction of HIV-1 release from infected cells. We hypothesize that HIV-1 infection coordinates cholesterol biosynthesis, trafficking, and transcription at least partially via SREBP, TFII-I and NPC-1. Novel chemicals which target this pathway and cholesterol homeostasis would be effective anti-HIV-1 therapeutics.

Minisymposium 1: Cell Biology of the Immune System (10-15)

10

Mechanisms of Immunological Synapse Formation.

M. Dustin, S. Vardhana, R. Varma; Skirball Institute of Biomolecular Medicine, New York University School of Medicine, New York, NY

Sensing foreign antigens in the immunological synapse (IS) involves T cell receptor (TCR) microclusters that initiate signaling in response to MHC-peptide ligands and then converge into a central supramolecular activation cluster (cSMAC). Before TCR signals are terminated via receptor degradation in the cSMAC, TCR-MHCp interactions within F-actin independent clusters are sorted from CD28-CD80 interactions. We implicate an essential role for endosomal sorting complexes required for transport (ESCRT) and EHD-3 in these distinct signal processing steps in the IS. Using siRNA technology in primary antigen specific T cells, we show that ESCRT-I component TSG101 is required for cSMAC formation, TCR signal termination, TCR degradation, and segregation of stable TCR-MHC-peptide complexes from dynamic CD28-CD80 complexes. ESCRT-III component VPS24 is only required for TCR degradation. EHD-3 silencing caused TCR to accumulate outside the cSMAC implicating a role for TCR recycling during activation. Less avid agonists MHCp induced CD80 and F-actin dependent TCR microclusters that disassociated in the F-actin poor center of the IS without recruiting ESCRT machinery. These results support a two-compartment cSMAC that segregates persistent TCR complexes for degradation from dynamically signaling costimulatory complexes. The immunological synapse contains spatially defined checkpoints that distinguish between stable complexes that require ESCRT action from unstable complexes that will spontaneously disassemble withotu ESCRT involvement. In T cells, ESCRT complexes appear to act at the plasma membrane on receptors that are engaged by ligands on other cells.

11

Regulation of Dendritic Cell Migration by CD74, the MHC Class II-associated Invariant Chain.

G. Faure-Andre[1], P. Vargas[1], M. Yuseff[1], J. Diaz[1], D. Lankar[1], F. Vascotto[1], G. Raposo[3], J. Boulanger[3], M. Bono[2], M. Rosemblatt[2], M. Piel[3], A. Lennon-Dumenil[1]; [1]Inserm U653, Institut Curie, Paris, France, [2]Universidad de Chile, Santiago, Chile, [3]UMR144, Institut Curie, Paris, France

The sampling of peripheral tissues is an essential function of dendritic cells (DCs) that is achieved by coupling efficient antigen uptake and processing to high motility. Here, we show that the MHCII-associated Invariant Chain (Ii or CD74), a known regulator of antigen processing, negatively regulates DC motility and in vivo migration. By using micro-fabricated channels that mimic the confined environment of peripheral tissues, we demonstrate that while wild-type DCs alternate high and low motility phases, Ii-deficient cells move in a uniform fast manner, indicating that Ii acts as a brake during DC locomotion. Our results further suggest that this unexpected property of Ii results from its association to the actin-based motor, Myosin II. We propose that the use of common mechanisms to regulate antigen processing and cell motility enables DCs to coordinate these two functions in time and space.

12

Fascin1 Is Critical for Dendritic Cell Motility and Morphology, but Is Dispensable for Neurite Extension and Mouse Development.

Y. Yamakita, S. Yamashiro; Mol. Biol. Biochem., Rutgers, The State Univ. of New Jersey, Piscataway, NJ

Fascin1, an actin-bundling protein, has been demonstrated to be critical for filopodia formation in cultured cells, and thus is believed to be vital in motile activities including neurite extension and cell migration. Consistent with this notion, fascin1 is highly expressed in specific types of cells including neuronal and glial cells and antigen-presenting, mature dendritic cells (DCs), which show many membrane protrusions such as filopodia. To test whether fascin1 plays such essential roles within a whole animal, we generated and characterized fascin1-deficient mice. Unexpectedly, fascin1-deficient mice are viable and fertile with no apparent developmental defect. Fascin1-deficient,

dorsal root ganglion neurons are able to extend neurites as well as those from wild-type mice, although fascin1-deficient growth cones are smaller and exhibit fewer and shorter filopodia than wild-type counterparts. Fascin1-deficiency, however, profoundly affects the morphology and motility of antigen-presenting, mature dendritic cells (DCs). Fascin1-deficient DCs exhibit fewer veil-like membrane protrusions with poor dynamics, and show significantly reduced migratory ability. Importantly, they show lower cortical tension than wild-type DCs. The lower cortical tension is consistent with localization of fascin1 at the cortex of mature DCs, a localization that has not been observed with other types of cells. These results reveal the physiological function of fascin1 in DC motility: fascin1 forms actin networks on the cortex, increasing the cortical tension and thereby enhancing membrane protrusive dynamics.

13

Coronin-1A Is Mutated in a Thymic Egress Defective Mouse Strain and a T-B+NK+ SCID Patient.

J. Cyster[1], L. Shiow[1], D. Roadcap[4], S. Watson[1], I. Grigorova[1], C. Goodnow[3], J. Bear[4], J. Puck[2]; [1]Department of Microbiology and Immunology, University of California, San Francisco/HHMI, San Francisco, CA, [2]Pediatrics, University of California, San Francisco, San Francisco, CA, [3]John Curtin School of Medical Research, The Australian National University, Canberra, ACT, Australia, [4]Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Mice carrying the recessive ptcd (peripheral T cell deficiency) locus have a block in thymic egress but the mechanism responsible is undefined. We find that ptcd T cells have an intrinsic migration defect, impaired lymphoid tissue trafficking, and irregularly shaped protrusions. The ptcd locus contains an E26K point mutation within the actin regulator Coronin-1A that enhances Arp2/3 inhibition, but mislocalizes the protein from the leading edge of migrating T cells. Discovery of another Coronin-1A mutant during an ENU-mutagenesis screen for T-lymphopenic mice prompted us to identify an atypical T-B+NK+ SCID patient with Coronin-1A deficiency. These findings establish a role for Coronin-1A in T cell egress, identify a surface of coronin involved in Arp2/3 regulation, and reveal actin regulation as a biological process defective in SCID.

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Concentration Thresholds for Signaling during Fcγ Receptor-mediated Phagocytosis.

Y. Zhang[1], A. Hoppe[2], J. A. Swanson[2,1]; [1]Biophysics, University of Michigan, Ann Arbor, MI, [2]Microbiology and Immunology, University of Michigan, Ann Arbor, MI

The zipper model of Fcγ Receptor (FcR)- mediated phagocytosis states that particle-bound IgG molecules interact with FcR in an ordered progression as the phagocytic cup advances over a particle, with each ligated receptor generating signals autonomously. However, recent studies suggest that signaling during phagocytosis may be integrated over the forming phagosomes, possibly through generation of 3' phosphoinositides (3'PIs). This study measured the magnitudes of FcR-generated signals in macrophage phagosomes as a function of ligand (IgG) density on 5.6μm diameter microsphere particles. A ratiometric fluorescence imaging method was developed to measure the quantity of YFP-labeled signaling molecules recruited to forming phagosomes. During phagocytosis, microspheres with high ligand densities recruited more YFP-Syk kinase and YFP-P85 protein (type I PI 3-kinase) than microspheres with low ligand densities, indicating that the magnitudes of early stages of FcR signaling were proportional to ligand density. In contrast, YFP-AktPH, which binds to PI(3,4,5)P3 and PI(3,4)P2 and YFP-Tapp1PH, which binds to PI(3,4)P2 only, were recruited to similar levels during phagocytosis of microspheres with high or low ligand density. YFP-AktPH recruitment also indicated PI(3,4,5)P3 concentration thresholds for completing phagocytosis. Microspheres with very low ligand densities bound to macrophages and stimulated recruitment of YFP-actin and formation of shallow phagocytic cups, but showed only low levels of YFP-AktPH recruitment and failed to complete phagocytosis. Similarly, recruitment of YFP-PKCε to phagocytic cups also showed evidence of FcR signal integration and 3'PI concentration-dependent thresholds for recruitment to phagocytic cups. These studies indicate that late FcR signals are not strictly autonomous, but rather are integrated by 3'PIs in membranes of phagocytic cups.

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Circadian Rhythm and Immunity in Drosophila: A Model for Neuroimmune Communication.

M. Shirasu-Hiza, D. Schneider; Microbiology and Immunology, Stanford University, Stanford, CA

We have demonstrated a functional and bi-directional relationship between circadian rhythm and innate immunity in Drosophila melanogaster. Circadian rhythm is known to affect a wide range of vertebrate physiologies, including many parameters of immune function. For example, in mice, hematopoietic stem cell release and the phagocytic activity of macrophages and neutrophils oscillate with circadian rhythm. We set out to ask if immunity and circadian rhythm interact in Drosophila. We found that animals infected with bacterial pathogens lose their circadian rhythm. Conversely, animals that lack circadian rhythm have dramatically altered immunity against infection. These results provide the first demonstration that circadian proteins regulate the immune response to bacterial pathogens. Further studies revealed that circadian regulation of immunity occurs during adulthood and involves at least two cell types: circadian clock neurons, located in the brain; and hemocytes, which phagocytose bacteria and foreign material. We found that, in wild-type flies, phagocytic activity oscillates with ciradian rhythm and that circadian mutants have defects in phagocytic hemocyte activity. We are currently investigating the molecular mechanisms underlying circadian regulation of phagocytosis, a paradigm for understanding neuronal regulation of the immune system. Because innate immune responses like phagocytosis by leukocytes are implicated not only in clearing bacterial infection but also in a growing number of diseases, including cancer and neurodegenerative disorders, information about circadian regulation of innate immunity could impact a broad spectrum of human health issues.

Minisymposium 2: Centrosomes and Cilia (16-21)

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Cryo-electron Tomography Reveals New Structures in the Flagellar Axoneme.

T. Heuser[1], M. Raytchev[1], J. Krell[1], M. Porter[2], D. Nicastro[1]; [1]Biology, Brandeis University, Waltham, MA, [2]Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN

Cilia and flagella are highly conserved microtubule-based organelles that perform critical motile or sensory functions in eukaryotic cells [reviewed in 1]. Despite their many different roles, almost all cilia and flagella share an identical core structure [reviewed in 2]. While significant progress has been made on understanding how the flagellar beat is generated, less is known about the components and mechanisms that regulate beat formation. Genetic analysis in *Chlamydomonas* has identified the dynein regulatory complex (DRC) as a key intermediate in the regulation of flagellar motility (reviewed in [3]), but the details of its structural organization are not well understood. Several DRC mutants with defects in beat regulation have been identified, leading to the discovery of at least 7 DRC components (reviewed in [3]). We are using cryo-electron tomography of intact and rapidly frozen *Chlamydomonas* axonemes in combination with image processing to gain new views into the 3D structure and molecular organization of the DRC *in situ* [4]. Recent improvements in the particle averaging software PEET [4,5] and increased numbers of averaged particles have allowed us to visualize the flagellar structure at 3-4 nm resolution. By comparing tomograms of flagella from wild type and several DRC mutants we localized key components of the DRC in unprecedented detail, including their spatial relationship to the nexin link. Our data show the distinct locations of structural defects in *pf2* and *pf3* mutant flagella as well as additional DRC densities not previously characterized. Our results also provide high resolution structures of other key complexes, such as the radial spokes, MIPs (microtubule inner proteins), and outer and inner dynein arms. This work will give novel insights into the 3D structure of the DRC at molecular resolution, and will contribute to a better understanding of how the DRC subunits interact and function to regulate flagellar bending (NIH funded). References: [1] Pan *et al*. (2005) Lab Invest 85: 452; [2] Porter & Sale (2000) J Cell Biol 151: F37; [3] Wirschell *et al*., In: The Chlamydomonas Sourcebook, ed. Witman, *in press*; [4] Nicastro *et al*. (2006) Science 313: 944; [5] PEET package @ bio3d.colorado.edu.

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Basal Body and Ciliary Proteins Affect Microtubule Stability.

S. Dutcher, H. Lin, J. Esparza, M. Miller; Department of Genetics, Washington University School of Medicine, St. Louis, MO

Mutations with basal body integrity defects (bld2, bld10, uni3) in Chlamydomonas confer increased sensitivity to the microtubule-depolymerizing drug, Taxol. This phenotype, in part, results from the loss or abnormal localization of the p80 subunit of the microtubule severing protein, katanin, in bld2, bld10, and uni3 cells. Proper katanin p80 localization is dependent on the presence of triplet microtubules in the basal body. Increased supersensitivity to Taxol is also conferred by mutations in the katanin p80 subunit, which is encoded by the PF15 gene (Dymek et al. 2004). To identify new mutants with basal body and microtubule defects, we screened for Taxol sensitive strains. We identified an insertional mutation in a predicted NimA-like kinase or Nek gene on linkage group I of Chlamydomonas (CNK10). Nek kinases have been implicated in ciliary, centrosomal and cell cycle functions from humans to Chlamydomonas. The closest mammalian homolog to CNK10 is Nek1; mutations in the mouse gene produce recessive polycystic kidney disease, bile duct dysplasia and male sterility (Upadhya et al., 2000 PNAS, 97:217). At the N-terminus, the predicted Cnk10 protein has a conserved kinase domain encoded by the first seven exons. This domain is 53% identical and 68% similar to the kinase domain of human Nek1. The insertional mutant results in a truncated mRNA that retains the kinase domain. The insertional cnk10 and an RNA interference strains reduce mRNA levels by ~4-fold and ~8-fold, respectively. The cnk10 strains also have a flagellar motility defect. They assemble two flagella, but generally only one flagellum beats. The cnk10 mutant is also defective in ciliary signaling; it shows a failure to complete mating. This phenotype is similar to PKD2 RNA interference strains (Huang et al., 2007). This NimA like kinase may regulate multiple genes involved in ciliary signaling and function and in microtubule dynamics.

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A Novel BBSome Subunit Regulates Ciliogenesis, Microtubule Stability and Acetylation.

A. Loktev[1], Q. Zhang[2], J. S. Beck[2], C. Searby[2], T. E. Scheetz[2], F. Bazan[1], D. Slusarski[3], V. C. Sheffield[2], P. Jackson[1], M. V. Nachury[4]; [1]Genentech, Inc., South San Francisco, CA, [2]Department of Pediatrics and Howard Hughes Medical Institute, University of Iowa, Iowa City, IA, [3]Department of Biology, University of Iowa, Iowa City, IA, [4]Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA

Bardet-Biedl syndrome (BBS) is a pleiotropic disorder affecting multiple organs and tissues by compromising function of the primary cilium. We have recently shown that seven highly conserved BBS proteins are present in a stable complex, the BBSome. Functionally, this complex is involved in membrane trafficking to and inside the primary cilium through its association with the GTP exchange factor for the small GTPase Rab8. We will present our initial discovery and extensive characterization of a novel BBSome subunit, that we named BBIP10. Similar to all other BBSome subunits, BBIP10 localizes to the primary cilium in fibroblasts, BBIP10 is present exclusively in the genomes of ciliated organisms and BBIP10 depletion in zebrafish yields phenotypes characteristic of BBS loss-of-function (reduction of the ciliated Kupffer's vesicle and defects in melanosome transport). Unexpectedly, BBIP10 was found to regulate the cytoplasmic microtubule cytoskeleton, a function unshared with any other BBSome subunits. Specifically, cells depleted of BBIP10 had significantly less polymerized tubulin and completely lacked acetylated microtubules while other tubulin modifications associated with stable microtubules remained unaffected. Strikingly, inhibition of the tubulin deacetylase HDAC6 rescues microtubule acetylation in BBIP10-depleted cells and BBIP10 physically interacts with HDAC6, thereby suggesting a role for BBIP10 in the regulation of HDAC6 activity. In summary, we have discovered a novel subunit of the BBSome that is required for ciliogenesis, but also appears to be essential for cytoplasmic microtubule stability and acetylation independently of the BBSome. We propose that BBSome-bound BBIP10 may function to couple acetylation of axonemal microtubules -by regulating HDAC6 activity- and ciliary membrane growth.

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Polo Kinase and Separase Define a Stepwise Mechanism for Mitotic Centriole Disengagement and Replicative Licensing in Human Cells.

M. Tsou[1,3], K. A. Yule[4], W. Wang[3], T. Stearns[1,2], P. V. Jallepalli[4]; [1]Dept. of Biology, Stanford University, Stanford, CA, [2]Dept. of Genetics,

Stanford University Medical School, Stanford, CA, [3]Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY, [4]Molecular Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY

The centrosome duplicates once per cell cycle in most dividing cells. The duplication process involves the alternating assembly and disengagement of centriole pairs at the core of each centrosome. Based on studies with Xenopus egg extracts, we proposed that separase-dependent centriole disengagement at anaphase licenses centrosomes for duplication in the next cell cycle. Here we use genetics and chemical biology to test this hypothesis in intact human cells. Homozygous deletion of the locus encoding separase caused a strong block to centriole disengagement during mitotic exit and inhibited timely assembly of new centrioles during the following S phase. Surprisingly, most of these persistent engagements were eventually dissolved before cells entered the next mitosis, implying the existence of an additional pathway that destabilizes centriole engagement. Using highly specific inhibitors, we identified Polo-like kinase 1 (Plk1) as an essential component of this second pathway, and further mapped its execution point vis-à-vis disengagement to late G2 or early M phase, well upstream of separase activation at anaphase onset. Crucially, when cells exited mitosis in the absence of both separase and Plk1, centriole disengagement failed completely, and subsequent centriole duplication in interphase was also prevented. Further, we show that disengagement could be restored through expression of wild-type but not protease-dead separase. These results indicate that the catalytic activities of Plk1 and separase cooperate to license centrosome duplication during M phase, analogous to their roles in removing cohesin from chromosomes.

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The Role of the Exocyst and Rab GTPases in Ciliogenesis.

X. Zhang[1], A. Knoedler[1], J. Zhang[1], Y. Zhao[1], J. Perenan[2], W. Guo[1]; [1]Biology, University of Pennsylvania, Philadelphia, PA, [2]Institute of Biotechnology, University of Helsinki, Helsinki, Finland

Primary cilia are centriole-derived, microtubule-based membrane projections found at the surface of many cell types. Primary cilia serve important sensory and signaling functions through the membrane proteins localized on their surface. Defects in primary cilia formation have been implicated in a number of genetic disorders such as Bardet-Biedl syndrome (BBS) and polycystic kidney disease (PKD). Recently, a number of studies have indicated that polarized vesicular transport is essential for ciliogenesis. At the molecular level, the players in the secretory pathway that mediate cilia formation and how they are regulated during ciliogenesis are unclear. The exocyst is an evolutionarily conserved octameric protein complex consisting of Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Exo70 and Exo84. The exocyst coordinates with cytoskeleton in the targeting of secretory vesicles to the plasma membrane for exocytosis, a process that allows protein incorporation at specific domains of the cell surface. Using immunofluorescence microscopy, we found that the exocyst co-localizes with gamma-tubulin at the basal body area of the cilium. Inhibition of the exocyst function in cells by RNA interference (RNAi) or dominant-negative exocyst mutants led to defective cilia formation. We have also found that the exocyst component Sec15 interacts with Rabin8, the guanine nucleotide exchange factor for Rab8 that associate with BBSome during ciliogenesis. In addition, the GTP-bound form of Rab11 directly interacts with Rabin8, suggesting that a cascade of Rab GTPases activation is involved in the control of membrane traffic from recycling endosomes to the plasma membrane. Disruption of this cascade or its connection with the exocyst in cells blocks cilia formation. Our results suggest that the exocyst complex and the Rab GTPases play essential roles in ciliogenesis by regulating membrane traffic to cilia.

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Centrosome Amplification Can Initiate Tumourigenesis in Drosophila.

R. Basto[1], K. Brunk[2], T. Vinadogrova[3], N. Peel[4], A. Franz[2], A. Khodjakov[4], J. Raff[2]; [1]Institut Curie, CNRS, Paris, France, [2]The Gurdon Institute, University of Cambridge, Cambridge, United Kingdom, [3]Wadsworth Center, New York State Dept. of Health, Albany, NY, [4]NIDDK, NIH, Bethesda, MD

Theodor Boveri first proposed that centrosome amplification could drive chromosomal instability and so tumourigenesis nearly 100 years ago. To test this hypothesis, we generated stable Drosophila lines that overexpress the centriole duplication protein Sak/Plk4. These flies have extra centrosomes in ~60% of their somatic cells. Although most cells with extra centrosomes initially form multipolar spindles, these invariably resolve into a bipolar spindle as most of the extra centrosomes become clustered at one of only two spindle poles, while those that are not clustered at a pole appear to be partially inactivated and so do not efficiently organise an extra spindle pole. Thus, there is only a very moderate increase in chromosomal instability in flies with extra centrosomes (from ~0.5% to ~1.5%), and these flies are viable and fertile and can maintain a stable diploid genome over many generations. The asymmetric division of the larval neuroblasts (stem cell-like progenitors that generate the cells of the adult nervous system), however, is compromised in the presence of extra centrosomes, and ~10% of these cells divide symmetrically — leading to an expansion of the neuroblast pool. Moreover, larval brain cells with extra centrosomes can generate metastatic tumours when transplanted into the abdomens of wild type hosts, something that never occurs when normal brain cells are transplanted. Thus, centrosome amplification appears to be sufficient to allow cells to initiate tumour formation in this system, although the mechanism remains uncertain.

Minisymposium 3: Endo- and Exocytosis (22-27)

22

Real-time Visualization of Membrane Fission Establishes Dynamin as the Minimal Fission Apparatus.

T. Pucadyil, S. Schmid; The Scripps Research Institute, La Jolla, CA

The GTPase dynamin assembles into a collar-like structure at the necks of budded vesicles in vivo where it is thought to play a role in membrane fission. Current models based on global nucleotide-driven conformational changes within preassembled dynamin have proposed that helical constriction, expansion or twisting generate forces necessary for membrane fission, but that dynamin alone is not sufficient. To reevaluate dynamin's function in membrane fission, we have developed a novel model membrane system of fluid supported bilayers on silica beads that allows us to both visualize and biochemically measure vesicle formation. Contrary to current models, we find that dynamin and

GTP hydrolysis are both necessary and sufficient to mediate membrane fission and vesicle formation. Importantly, fluorescently-labeled dynamin added in the continuous presence of GTP, cooperatively organizes into short 'collar-like' structures at the necks of large constricted buds. This intermediate persists for differing time periods before its partial disassembly coincident with membrane fission and release of the vesicle. These results clearly establish that dynamin constitutes the minimal protein machinery for membrane fission.

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Membrane Fission: Lessons from Lipid Nanotubes and Dynamin.

P. Bashkirov[1,2], A. Sergey[1,2], S. Schmid[3], J. Zimmerberg[1], V. Frolov[1]; [1]Program on Physical Biology, Eunice Kennedy Shriver National Institute of Child Health and Human Development, NIH, Bethesda, MD, [2]A.N. Frumkin Institute of Physical Chemistry and Electrochemistry, Moscow, Russian Federation, [3]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA

Formation of a transport vesicle is finalized by scission of a thin neck connecting the vesicle with a cellular membrane. To avoid leakage of the vesicle contents, the neck should be carefully restructured to achieve merger of the inner leaflet of the neck membrane. To understand how proteins can arrange such non-leaky membrane rearrangements, we reconstituted fission of lipid nanotubos by dynamin, the key component of the membrane fission machinery in cells. Using electrophysiological measurements we proved that the dynamin-induced membrane fission is leak-free and identified two main steps in the fission process. First, dynamin self-assembles on the nanotube membrane generating high membrane curvature. Second, cooperative hydrolysis of GTP triggered by the dynamin assembly leads to curvature instability and fission. The GTP hydrolysis does not cause additional curvature stress or generation of substantial membrane tension. Fission follows hydrolysis only when the dynamin scaffold brings the encaged nanotube close to the point of curvature instability. At this point fusion of inner monolayer of the neck becomes energetically favorable, according to our theoretical calculations. On rigid membrane templates, GTP hydrolysis causes disassembly of the dynamin scaffold followed by relaxation of membrane curvature. Thus, dynamin functions as a GTP-dependent catalyst of membrane fission whose activity critically depends on the mechanical and geometrical parameters of the membrane.

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Lack of Dynamin Unmasks a Powerful Actin-dependent Plasma Membrane Tubulating Activity of BAR Proteins.

S. Ferguson[1], A. Raimondi[1], S. Paradise[1], H. Shen[1], O. Destaing[1], K. Mesaki[2,1], O. Cremona[3], P. De Camilli[1]; [1]HHMI/ Dept of Cell Biology, Yale University School of Medicine, New Haven, CT, [2]Department of Neuroscience, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayamashi, Japan, [3]IFOM, the FIRC Institute for Molecular Oncology Foundation, Università Vita - Salute San Raffaele, Milan, Italy

Mammalian genomes encode 3 homologous large dynamin GTPases that vary in their expression patterns but share binding to a variety of membrane deforming and actin regulatory proteins. Extensive evidence based on pharmacology, dominant-negative mutants, RNAi knockdowns and temperature-sensitive alleles implicates dynamin in the fission reaction of endocytosis. However, a precise understanding of how dynamin and its binding partners cooperate in their actions on the membrane and on the actin cytoskeleton to culminate in vesicle fission is still missing. To elucidate the essential function(s) of dynamin and the specific functions of dynamin isoforms, we have generated knockout mice for all three dynamin genes. While the ubiquitous dynamin 2 isoform is essential for embryonic development, dynamin-null fibroblasts from conditional KO mice are surprisingly viable and exhibit grossly normal morphology. The most striking defect is the accumulation of clathrin coated pits (CCPs) at the end of long, narrow, actin embedded, tubular invaginations of the plasma membrane. This proves that dynamin is not required for early stages in clathrin-mediated budding, but that it acts to sever a vesicle neck generated by other factors. In the absence of dynamin, the misregulated activity of endocytic BAR domain containing proteins may generate the abnormally long and narrow tubules. In support of this hypothesis, BAR proteins such as endophilin 2 and sorting nexin 9 were highly concentrated along with N-WASP and Arp2/3 at the sites where these invaginations occur. Acute actin depolymerization abolished the long tubules and dispersed endophilin 2 and Arp2/3, leaving the CCPs connected to the plasma membrane by relatively short, wide necks. These findings provide genetic evidence for a coordinated action of BAR proteins and actin at the necks of CCPs prior to, and independent of, the actions of dynamin. They further underscore an important role for dynamin in controlling actin dynamics at the plasma membrane.

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The Recycling Endosome as a Hub for Polarized Secretion.

J. Stow, L. Hammond, J. Kay, R. Misaki, T. Taguchi, N. Hamilton; Institute for Molecular Bioscience, University of Queensland, St. Lucia, QLD, Australia

Recycling endosomes (RE) typically recycle endocytosed cargo back to the cell surface. More recently, secretory traffic from the Golgi complex destined for the cell surface has also been shown to pass through RE. Diverse exocytic cargo including cell adhesion proteins and cytokines are transported from the trans-Golgi network to RE. Proteins emerging from RE are delivered selectively to sites on the plasma membrane and sorting within RE is responsible for this polarized trafficking and secretion. A novel quantitative image analysis technique has allowed us to measure the relative overlap of multiple cargo proteins moving through RE in live cells. This analysis has been applied to transferrin coexpressed with the cytokines IL-6 and TNF as mixed cargo and to cells coexpressing resident proteins of RE including VAMP3 and multiple Rabs. The results show that there are distinct subcompartments of RE that allow segregation of cargo within RE and selective exit of different cargo. RE have been compared between cell types to further define subcompartments as a common mechanism for sorting and polarized trafficking.

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Functional Delineation of Complexin's Clamping Mechanism in Calcium-regulated Membrane Fusion In Vitro.

C. Giraudo, A. Garcia-Diaz, W. S. Eng, T. J. Melia, J. E. Rothman; Physiology & Cellular Biophysics, Columbia University, New York, NY

In regulated exocytosis, the core membrane fusion machinery proteins - the SNARE proteins - are assisted by a group of regulatory factors in order to couple membrane fusion to an increase of intracellular calcium ion (Ca2+) concentration. Complexin-I and Synaptotagmin-I have

been shown to be key elements for this tightly regulated process. Many studies suggest that Complexin-I can arrest the fusion reaction and that Synaptotagmin-I can release the Complexin-I blockage in Calcium dependent manner. Although the actual molecular mechanism by which they exert their function is still unknown, recent in vivo and in vitro experiments postulate that domains of Complexin-I produce different effects on neurotransmitter release. Herein, by using an in vitro flipped SNARE cell fusion assay we have identified and characterized the minimal functional domains of Complexin-I necessary to couple Calcium and Synaptotagmin-I to membrane fusion. Moreover, combining results from the crystal structure of the Complexin-1/SNAREpin complex, from mutagenesis experiments and from functional studies using the flipped-SNARE cell fusion assay we have been able to provide for first time direct evidence of the mechanism(s) by which Complexin may exert its clamping effect on calcium-regulated membrane fusion.

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Partial Release (Kiss-and-Run Fusion) Is the Predominant Mode of Exocytosis of Post-Golgi Vesicles.

J. K. Jaiswal[1], V. M. Riviera[2], S. M. Simon[1]; [1]Laboratory of Cellular Biophysics, The Rockefeller University, New York, NY, [2]ARIAD Gene Therapeutics, Inc, Cambridge, MA

The final step of exocytosis of hormones and neurotransmitter is regulated by the formation and expansion of the fusion pore. This allows the nature and extent of cargo released by individual vesicles to be controlled. To examine if similar control exists during constitutive secretion of biosynthetic cargo we established a technique for simultaneously monitoring the delivery of luminal and membrane cargoes from individual post-Golgi vesicles. We observe varying degree of release of the luminal and membrane proteins ranging from partial to complete release, with partial release being the predominant fate. We will present the characterization of these release events and manipulations the molecules that modify the extent of cargo release by the post-Golgi vesicles

Minisymposium 4: Impact of Protein Modifications on Cell Biology (28-33)

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KSR2 Is a Calcineurin Substrate That Promotes ERK Cascade Activation in Response to Calcium Signals.

M. Dougherty[1], S. Specht[1], M. Zhou[2], T. Veenstra[2], D. Morrison[1]; [1]Laboratory of Cell and Developmental Signaling, National Cancer Institute-Frederick/National Institutes of Health, Frederick, MD, [2]Laboratory of Proteomics and Analytical Technologies, SAIC-Frederick, Inc, Frederick, MD

Protein scaffolds have emerged as important regulators of MAPK cascades, facilitating kinase activation and providing crucial spatio/temporal control to their signaling outputs. Members of the KSR family serve as conserved ERK signaling scaffolds in many animal species, and the presence of two mammalian KSR proteins suggests that they might mediate distinct scaffold functions in different contexts. We explored this possibility by utilizing a proteomics approach to compare KSR1- and KSR2-binding partners. We find that both KSR1 and KSR2 interact with the core kinase components of the ERK cascade and facilitate signaling in response to RTK activation. Strikingly, we find that the protein phosphatase calcineurin selectively interacts with KSR2 via an LxVP motif. We demonstrate further that in response to Ca2+ signals, calcineurin dephosphorylates KSR2 on specific residues, thus regulating KSR2 localization and activity. These findings identify KSR2 as a Ca2+-regulated ERK scaffold and reveal a new mechanism whereby Ca2+ impacts Ras/ERK pathway signaling.

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Identification of the First Mammalian α-N-methyltransferase, NR-MT, Which Methylates the Ran Exchange Factor, RCC1.

C. Schaner Tooley, J. Petkowski, I. Macara; University of Virginia, Charlottesville, VA

N-terminal methylation of the α-amino group was first discovered as a posttranslational modification over thirty years ago. Since then, several proteins have been found to be modified in this way, predominantly in bacteria but including, most recently, the Ran quanine nucleotide-exchange factor, RCC1. Inability of RCC1 to be α-N-terminally methylated results in its reduced affinity for DNA and subsequent mitotic defects, including supernumerary spindle poles. Sequence comparisons of the α-N-methylated proteins in eukaryotes have revealed a cleavage of the N-terminal methionine and a resulting X/P/K N-terminal consensus sequence. However, nothing is known about the enzyme that methylates this motif. We now report the identification of the first human X/P/K N-terminal methyltransferase, NR-MT. NR-MT is able both to di-and trimethylate RCC1 on its N-terminal serine. Surprisingly, NR-MT does not contain a SET domain (found in lysine N-methyltransferases), but the enzyme has been highly conserved throughout evolution. Systematic mutation of the first amino acid of RCC1 has allowed for an expanded definition of the consensus sequence and the identification of many other potential targets. These targets are involved in a wide variety of cellular processes, including transcription, cell-cycle progression, centromere assembly, cell migration and neurofibrillary tangle formation. The identification of this novel enzyme now allows us to study a new layer of protein regulation during these processes that has hereto now been predominantly undeveloped. Supported by grants GM-50526 (to IM) and GM-078862 (to CS)

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Arginylation Regulates Cytoskeleton Architecture by Modulating Intracellular Actin Polymer Levels and Differential Binding of Capping and Severing Proteins to the Actin Filaments.

S. Saha[1], F. Zhang[1], T. Svitkina[2], F. Korobova[2], A. Kashina[1]; [1]Dept of Animal Biology, University of Pennsylvania, Philadelphia, PA, [2]Department of Biology, University of Pennsylvania, Philadelphia, PA

Arginylation is a poorly understood posttranslational modification that is essential for embryogenesis. Our recent work demonstrated that actin arginylation regulates lamella formation in motile fibroblasts. We have also identified a number of actin cytoskeleton associated proteins and several other groups of proteins which undergo arginylation in vivo. However the role of these arginylated proteins or the molecular mechanisms by which arginylation regulates cellular functions are unknown. Here we address this question by investigating the biochemical properties and the structural organization of actin filaments in vivo in wild type and arginylation-deficient arginyltransferase (Ate1) knockout cells. Remarkably, we find that Ate1 knockout results in a dramatic (more than 2.5 fold) reduction of the actin polymer

levels in vivo accompanied by a corresponding increase in the monomer level. Electron microscopy of the platinum replicas reveals severe impairments of the cytoskeletal organization throughout the cell and at the leading edge, increased number of filament ends in the lamellipodial actin network and dramatic impairments in filopodia formation at the leading edge of Ate1 knockout cells. Actin filaments polymerized in extracts of Ate1 knockout cells show altered interaction with several associated proteins, including a marked increase in binding of capping protein and twinfilin and a decreased binding of gelsolin. Purified non-arginylated actin has altered polymerization properties and forms abnormal non-filament assemblies at physiological concentrations, suggesting that arginylation regulates actin's ability to form filaments. Taken together, our data suggest that arginylation regulates actin ultrastructure through interconnected mechanisms that involve actin polymerization, combined with the modulation of binding and function of proteins that together ensure the proper assembly and dynamics of intracellular cytoskeletal networks.

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SUMO in Ras Signaling and Cell Cycle Regulation.

M. Nie[1], Y. Xie[1], J. A. Loo[1,2], A. J. Courey[1]; [1]Chemistry and Biochemistry, UCLA, Los Angeles, CA, [2]Department of Biological Chemistry, David Geffen School of Medicine, UCLA, Los Angeles, CA

Post-translational protein modification by SUMO is essential for viability and growth in many organisms. To elucidate the functions of SUMO in multi-cellular development, we analyzed the phenotypes of *sumo* mutant embryos of *Drosophila melanogaster*. We observed a broad spectrum of defects, including eggshell ventralization, diverse embryonic patterning defects, and aberrant syncytial nuclear cycles. Since dorsoventral patterning of the eggshell depends on EGFR/Ras signaling, we employed cell culture assays to examine the role of SUMO in the Ras pathway and discovered that SUMO is required for robust activation of the Ras/MAPK pathway. Using a bacterial sumoylation assay, we found that Ras could be efficiently sumoylated in its hypervariable region. The nuclear cycle defects in the *sumo* syncytial embryo imply a role for SUMO in mitosis. Throughout mitosis, SUMO is associated with the pericentromertic regions of the chromosomes, and is also found at the spindle midbody late in mitosis, suggesting a dynamic role of sumoylation in the mitotic cycle. A requirement for SUMO in cell cycle progression was also demonstrated in cultured S2 cell and larval imaginal tissues by RNAi-mediated knockdown of SUMO. To identify sumoylation targets that might account for some of the roles for SUMO revealed by our genetic analysis, we developed an approach for tagging SUMO conjugates *in vivo*. Tagged SUMO conjugates were then purified from embryo extracts and identified by mass spectrometry. In addition to Ras, we found that several proteins involved in Ras signaling, as well as cell cycle regulation, including 14-3-3ζ, PCNA, RFC2, and Polo, were SUMO conjugation targets. We also found that SUMO partially colocalizes with Polo and the SUMO conjugating enzyme on the mitotic chromosomes, further suggesting Polo as a SUMO substrate during mitosis, with active sumoylation taking place on the mitotic chromosomes.

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Real-Time Editing of Parkin Ubiquitination by Ataxin-3 in Machado-Joseph Disease.

T. M. Durcan[1], M. Kontogiannea[1], T. Thorarinsdottir[1], A. Williams[2], H. L. Paulson[2], E. A. Fon[1]; [1]Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, QC, Canada, [2]Neurology, University of Michigan, Ann arbor, MI

Machado-Joseph's disease (MJD), the most common dominantly inherited ataxia worldwide, is caused by a polyglutamine (polyQ) expansion in the deubiquitinating enzyme ataxin-3. We show here that ataxin-3 deubiquitinates parkin, a Ub-ligase responsible for a familial form of Parkinson's disease in both *in vitro* ubiquitination reactions and *in vivo*. Remarkably, ataxin-3-mediated deubiquitination is coupled to parkin self-ubiquitination, such that ataxin-3 can hydrolyze Ub conjugates on parkin in "real-time" as they are being added, but not pre-assembled conjugates. Moreover, ataxin-3 preferentially cleaves K63-linked Ub on parkin, suggesting it shapes the architecture of Ub chains as it is being assembled by shifting the balance of parkin self-ubiquitination from K63 toward K48, thereby promoting proteasomal degradation of parkin. Moreover, polyQ-expanded ataxin-3 removes K63-linked Ub from parkin more efficiently than wild type and reduces parkin levels in vivo. Considering its neuroprotective role, decreasing parkin levels via ataxin-3-mediated deubiquitination provides a novel mechanism of neurodegeneration, potentially accounting for the parkinsonian features observed in MJD.

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Polysialic Acid of the Neural Cell Adhesion Molecule NCAM Is Essential for Kidney Maturation.

R. Gerardy-Schahn[2], B. Weinhold[2], C. Zuber[1], J. Roth[1]; [1]Dept. of Pathology, University of Zurich, Zurich, Switzerland, [2]Cellular Chemistry, Medical High School, Hannover, Germany

Previously, we reported specific cellular expression pattern of polysialylated neural cell adhesion molecule (NCAM) during inductive interactions in kidney development and proposed that α2,8-linked polysialic acid (poly Sia) is important in specific kidney maturation processes. The generation of NCAM-positive mice in which different polysialyltransferase genes were knocked-out has now permitted to analyze our proposal. We find that in kidneys of ST8Sia-II and IV double knock-out mice, poly Sia was undetectable in tissue sections by immunohistochemistry and in tissue homogenates by Western blot. The kidneys of ST8Sia-II and -IV double knock-out mice had a regular shape and histologically a regular principal architecture, but were only half the size of age-matched controls. The time period of postnatal nephrogenesis was extended. A detailed electron microscopic analysis revealed striking defects of the glomeruli, the filtration barrier of the nephron, in two weeks old animals. In contrast to age-matched controls, the glomeruli of double knock-out animals were smaller and compact and exhibited severe abnormalities. The glomerular capillaries, when present, possessed only a slit-like lumen due to endotheliosis. The capillary endothelial cells were highly immature and lacked their characteristic flat cytoplasma domains and their typical fenestrations. A non-fused, endothelial and podocyte-derived, double lamina densa instead of a single fused lamina densa of the glomerular basement membrane was observed. Importantly, the podocytes were immature since they lacked secondary foot processes and slit diaphragms. The proximal tubules were atrophic as manifestation of glomerular misfunction. In contrast to the double polysialyltransferase knockout animals, only a mild glomerular phenotype was observed in each of the single polysialyltransferase knock-out animals. The double polysialyltransferase knock-out glomerular phenotype could be rescued by additional NCAM knockout. Together, our data demonstrate the

essential role of poly Sia in kidney maturation and reveal that this unique and highly specific posttranslational modification orchestrates NCAM interactions in vivo during kidney maturation.

Minisymposium 5: Information Technology for Cell Biology (34-39)

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Interpreting Patterns of Cellular Heterogeneity.

S. J. Altschuler[1,2], E. Martinez[1,3], M. D. Slack[1,2], L. F. Wu[1,2]; [1]Department of Pharmacology, UT Southwestern Medical Center at Dallas, Dallas, TX, [2]Green Center for Systems Biology, UT Southwestern Medical Center at Dallas, Dallas, TX, [3]Hamon Center for Therapeutic Oncology Research, UT Southwestern Medical Center at Dallas, Dallas, TX

Phenotypic heterogeneity in cellular populations has been widely observed. However, interpreting possible roles of cellular heterogeneity in mammalian biology and disease is an exceedingly challenging problem due to the complexity of possible cellular phenotypes, the large dimension of potential perturbations, and the lack of methods for separating meaningful biological information from noise. Our objective is to develop analytical approaches for summarizing and interpreting heterogeneity that can be used to investigate changes due to physiological, pathological, or environmental perturbations. Our approach links high-content microscopy-based assays with image processing, statistical analysis, and machine learning algorithms. Our analysis platform extracts phenotypic measurements, such as cell morphology and the activation and co-localization patterns of cellular readouts, from large populations of individually identified cells. Algorithms, loosely inspired by automated face recognition, are used to characterize heterogeneous cellular populations as mixtures of phenotypically distinct subpopulations. These "subpopulation profiles" allow heterogeneity to be effectively summarized and compared across populations and conditions. We applied our subpopulation profiling approach to compare the heterogeneous effects of a collection of drugs applied to cancer cells. Clustering of the subpopulation profiles vectors revealed that similarity and dissimilarity of subpopulation profiles strongly matched similarity or dissimilarity of drug mechanism, and yielded highly accurate mechanistic classifications of drugs. Thus, we found that patterns of heterogeneous responses appeared to encode information about the mechanisms of cellular perturbations. Parameterizing heterogeneous responses to perturbations as ensembles of cellular stereotypes offers a framework to interpret the complexity of drug effects, and has the potential to identify subpopulations of physiological and clinical importance missed by population-averaged studies. We have begun to apply our methodology more broadly to interpret the role of heterogeneity in cancer, development, and cell motility.

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A High-Throughput Single-Cell Microfluidic Platform for Screening Dynamic Phenotypes.

R. Taylor[1,2], D. Falconnet[2], A. Niemisto[1], S. Ramsey[1], S. Prinz[1], I. Shmulevich[1], T. Galitski[1,2], C. Hansen[1,2]; [1]Institute for Systems Biology, Seattle, WA, [2]University of British Columbia, Vancouver, BC, Canada

Much of the biology of cells is fundamentally dynamical. Insights can be gained by addressing this reality directly, that is, by dynamically interrogating pathways and observing the responses of individual cells. Such data will be needed increasingly to serve as input and tests of mathematical models of cellular processes. We have developed high-throughput microfluidic technology for the study of pathway dynamics in single cells. We applied this technology to probe the dynamics of the budding yeast pheromone-response mating pathway when subjected to a combination of pathway mutations and time-varying stimulus (alpha factor) concentration. A combination of scalable microfluidic fluid control and time-lapse live-cell visible and fluorescent imaging was used to implement 256 simultaneous quantitative experiments. In each experiment, pathway output (a GFP pathway reporter), growth rate, and cell morphology were measured at single-cell resolution across 32 temporal sequences of alpha-factor concentration and in 8 mutant backgrounds. Using this high-throughput technology we conducted over 1000 live-cell experiments investigating the interplay of stimulus-concentration sequences and genetic perturbations on pathway response over time. Our analyses implicate specific pathway components in dynamical regulatory roles in changing environments. For example, the results suggest that the mating pathway maintains a memory of past stimulations, and that this depends on specific pathway components. Our results demonstrate the development and application of a high-throughput general-purpose microfluidic experimental platform.

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A Cellular Resolution Atlas of Gene Expression in Drosophila pseudoobscura Reveals Interspecies Variation in Embryonic Patterning.

A. DePace[1], C. Fowlkes[5], C. Luengo[7], S. Keränen[2], C. Henriquez[2], L. Simirenko[2], G. Weber[2], O. Rübel[6], M. Huang[6], J. Malik[3], M. Biggin[2], D. Knowles[2], M. Eisen[4,2]; [1]Systems Biology, Harvard Medical School, Boston, MA, [2]Genome Sciences, Lawrence Berkeley National Labs, Berkeley, CA, [3]Computer Science, University of California Berkeley, Berkeley, CA, [4]Molecular and Cellular Biology, University of California Berkeley, Berkeley, CA, [5]Computer Science, University of California Irvine, Irvine, CA, [6]Computer and Data Visualization Department, University of California Davis, Davis, CA, [7]Centre for Image Analysis, Uppsala University, Uppsala, Sweden

Understanding how gene regulatory networks evolve requires us to measure the functional consequences of even small changes in sequence. We have applied high-resolution microscopy and image processing methods to blastoderm embryos of *D. melanogaster* and *D. pseudoobscura* to determine the expression patterns of key transcriptional regulators and a subset of their targets in their native context at cellular resolution in 3D over the hour prior to gastrulation. These two species shared their last common ancestor nearly 30 million years ago, and comparative sequence analysis reveals a wide variety of changes in *cis*-regulatory elements. Our imaging techniques allow multiple types of statistically rigorous inter-species comparisons to be made, both between individual embryos and between composite multi-gene models, revealing widespread quantitative changes in expression patterns. We measure multiple types of gene-specific variation, including changes in spatial position, number of cells comprising a pattern, and the dynamics of expression. Our comparative analyses aim to put these differences in the context of complete developing embryos. Which changes are due to differences in the geometry of the embryos and which are due to genetic differences in the transcriptional networks? Furthermore, which are changes initiated by variation in the *trans*-network, and which are due to changes in how *cis*-regulatory sequences interpret that network? Differentiating these types of variation will allow us to interpret

which specific sequence changes have functional consequences for gene expression, and provide insights into the functional constraints under which *cis*-regulatory elements evolve.

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High-Content Image Analysis in Early Steps of Endocytosis.

D. Loerke[1], M. Mettlen[1], D. Yarar[2], D. Nunez[1], S. Schmid[1], G. Danuser[1]; [1]The Scripps Research Institute, La Jolla, CA, [2]The Whitehead Institute for Biomedical Research, Cambridge, MA

Clathrin-mediated endocytosis (CME) involves the assembly of clathrin-coated pits (CCPs) from cytosolic coat proteins, capture of transmembrane cargo molecules, invagination, and pinching off from the membrane as vesicles. In adherent cells, CME lends itself to high-resolution TIRF microscopy allowing the tracking of the full CCP maturation process. Some of the steps occur in a regime of low molecular numbers, making CCP maturation an intrinsically stochastic process. Furthermore, the distribution of the molecules may be spatially variable. To capture the full heterogeneity of this process, we have applied novel single particle tracking methods (Jaqaman et al., 2008) to provide a complete and unbiased inventory CCP motion and lifetimes. In combination with molecular perturbations we have used this assay to systematically probe the factors that regulate CCP assembly and maturation. Using the lifetime distributions of all visible CCPs and statistical model selection, we identified three CCP subpopulations with differential kinetic regulation, revealing an endocytic checkpoint in which cargo and endocytic adaptor molecules gate CCP maturation. Spatial analysis of CCP trajectories revealed that CCP maturation is highly localized. We propose an actin-mediated membrane matrix as the likely mediator of a molecular scaffold that organizes accessory factors required for CCP maturation. By correlative analysis of CCP maturation and actin speckle dynamics, we found that CCP internalization is preceded by depolymerization of actin filaments, suggesting that the actin cortex subadjacent to the plasma membrane has to locally soften to allow the passage of CCPs. Thus, image data modeling identified a dual role of actin in endocytosis, i.e. both as a positive (scaffold) and as a negative regulator (barrier). Our model reconciles the controversial outcomes of many biochemical studies of the effects of actin-modifying drugs on endocytosis. It also suggests that the observed burst of actin polymerization, which has been thought to provide force for CCP internalization, may constitute the refilling of the cortical cavity.

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Executable Cell Biology.

J. Fisher; Computational Biology, Microsoft Research Cambridge, Cambridge, United Kingdom

Computational modelling of biological systems is becoming increasingly important in efforts to better understand complex biological behaviours. 'Executable Biology' is a pioneering approach focused on the design of executable computer programs that mimic biological phenomena. While traditional mechanistic models in biology are usually described by diagrams (giving a fairly static picture of cellular processes), executable biology seeks to translate such static diagrams into dynamic models using formal computational methods that were originally designed for the construction and analysis of complex man-made systems (i.e., computers and computer programs). The resulting dynamic models can then be compared to real experimental data, revealing gaps in our understanding of the processes taking place, and leading to new discoveries in a wide variety of biological systems. The operational view in which such models are created is very suitable, in many cases, to the way biologists reason about biological phenomena, and it renders itself to a simple implementation of biological knowledge in the form of an executable model. In addition, these kinds of models can be used to test hypotheses that would fill in the missing pieces in silico before performing actual lab experiments, thus saving valuable laboratory time and resources for only the most promising research avenues. Several modelling efforts put the executable biology framework into practice (e.g., Boolean networks, Petri nets, process calculi, interacting state-machines) each with its strengths and weaknesses. In the talk, I will survey the main modelling efforts in this direction, demonstrate some of the 'success stories' of executable models in biological research (through models of cell fate determination and cell signalling crosstalk during animal development), and highlight some of the main challenges that executable biology poses for biological and medical research.

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On the Future of Models in Systems Biology.

W. Fontana[1], V. Danos[2], J. Krivine[1], J. Feret[1], R. Harmer[3], E. J. Deeds[1]; [1]Department of Systems Biology, Harvard Medical School, Boston, MA, [2]School of Informatics, University of Edinburgh, Edinburgh, United Kingdom, [3]Ecole Normale Superieure, Paris, France

Problem. Models are instruments for reasoning about how systems of molecular interactions give rise to coherent, adaptive, and evolvable behavior. Yet, molecular systems underlying cellular decision processes are inherently combinatorial. Moreover, empirical facts are scatterd across research communities, while being constantly updated. These challenges break the practice of traditional modeling techniques that originated and triumphed in physics. **Objective.** Our objective is to develop a rigorous, mathematically founded, but highly intuitive technology for building models capable of meeting these challenges. **Method.** Protein interactions (both factual and hypothetical) are represented by means of rules expressed in a formal language that captures a simple yet biologically meaningful level of abstraction. The use of a language and of rules stands in analogy to how knowledge is organized in organic chemistry. Models become easily updatable collections of empirical but executable facts that operate on a mixture of molecular agents. **Results.** The dynamics and causal structure of EGF, IGF, and mTOR signaling becomes amenable to exploration, despite the astronomic number of possible molecular species these systems can generate. Static proteomic maps depicting large-scale protein interactions at the domain level can now be fully animated. Automatic tools were developed for identifying causal relationships that exist between rules in a model, as well as extracting and sampling event structures that reveal causal pathways from an initial state of the system to the first appearance of any user-specified observable. Such formal pathways are not static, but exhibit adaptation to changes in system composition. **Conclusion.** Our framework suggests a concept of model akin to the concept of a program in computer science. However, the "programming language" in which models are written is one that directly represents molecular interactions. This enables a perspective for approaching biological systems through the lens of computer science.

Minisymposium 6: Interactions with the Cytoskeleton (40-45)

40

Functions and Mechanisms of Microtubule Plus-end Tracking Proteins (+TIPs).

H. Goodson; Department of Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN

Microtubule plus-end tracking proteins (+TIPs) dynamically track microtubule (MT) plus ends. This localization puts them in the right place to be master regulators of MT dynamics, but many aspects of +TIP function and mechanism remain obscure. In particular, the significance of the complex web of interactions between +TIPs and other parts of the cytoskeleton is only just beginning to be elucidated. This presentation will focus on our recent efforts to utilize a combination of approaches (biochemical, cell biological, and computational) to investigate two interacting +TIPs, CLIP-170 and EB1. Particular areas of focus include the activities of these proteins, the mechanisms for these activities, the structure/function relationships underlying these activities, and the significance of the interactions between these proteins.

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Molecular Mechanism of EB1-dependent Microtubule Tip Tracking.

S. Honnappa[1], S. Montenegro Gouveia[2], A. Weisbrich[1], N. Bhavesh[3], F. Damberger[3], H. Jawhari[1], I. Grigoriev[2], R. M. Buey[1], I. Jelesarov[4], F. Winkler[1], K. Wüthrich[3], A. Akhmanova[2], M. Steinmetz[1]; [1]Paul Scherrer Institute, Villigen PSI, Switzerland, [2]Erasmus Medical Center, Rotterdam, Netherlands, [3]ETH Zurich, Zurich, Switzerland, [4]University of Zurich, Zurich, Switzerland

Microtubule plus-end tracking proteins (+TIPs) play a major role in many essential cellular activities including chromosome segregation, cell polarization and migration, organelle transport, intracellular signaling, and tumorigenesis. End binding protein-1 (EB1) and its family members recently emerged as master regulators of +TIP interaction networks at microtubule ends. Two facts underline its central role. First, EB1 autonomously tracks growing microtubule tips independently of any binding partners. Second, the C-terminal moiety of EB1 encompassing the highly conserved EB homology (EBH) domain binds to an array of complex and functionally unrelated +TIPs. A major intensively investigated question is how EB1 binds and controls the localization to microtubule ends of diverse +TIPs. Using structural, biophysical, biochemical and cell biological methods we found that a short motif, SxIP, within basic and serine-rich sequence regions is used by diverse +TIPs, including the kinesin MCAK, the transmembrane protein STIM1, and the tumor suppressor APC for localization to growing microtubule ends in an EB1-dependent fashion. Using NMR and X-ray crystallography we establish that EBH domains are SxIP motif recognition domains. By combining in vitro reconstitution with live cell experiments we demonstrate that the EB1-SxIP interaction controls the targeting of +TIPs to growing microtubule ends, a process that can be negatively regulated by phosphorylation. Remarkably, a short, 12-residue peptide encompassing SxIP is sufficient for tracking growing microtubule ends. Our findings define a universal 'microtubule tip localization signal' (MtLS) and provide molecular insights into the master role of EB proteins in integrating functionally diverse binding partners within dynamic +TIP networks. They allowed us to predict MtLSs in two functionally unrelated proteins and we anticipate that our approach is generally applicable to many other +TIPs. Furthermore, our findings offer a basis to systematically dissect the role of EB-association and microtubule plus-end tracking in various cellular processes. Supported by the Swiss National Science Foundation and the National Center of Competence in Research Structural Biology program.

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Cytokinesis in Caulobacter: Identification of Novel FtsZ-binding Proteins.

E. Goley[1], J. Werner[2], Z. Gitai[2], L. Shapiro[1]; [1]Developmental Biology, Stanford University, Stanford, CA, [2]Molecular Biology, Princeton University, Princeton, NJ

FtsZ, a bacterial tubulin relative, is essential for cytokinesis in the vast majority of bacterial species. It polymerizes at midcell, serves as a scaffold for recruitment of the cell division machinery (divisome), and may exert constrictive forces to drive invagination of the cell envelope. However, the molecular mechanisms underlying its function are unknown. As FtsZ is likely to be assisted by interacting partners, we sought to identify FtsZ-binding proteins in the dimorphic α-proteobacterium Caulobacter crescentus. To do this, we developed an assay in which we overproduce a GTPase-defective mutant of FtsZ, causing cells to adopt a distinct filamentous morphology wherein slender constrictions separate the two cell bodies. Known FtsZ-binding proteins co-localized with FtsZ in the extended constrictions of mutant cells, whereas divisome proteins that do not bind to FtsZ were diffuse. To identify novel FtsZ-binding proteins we used this assay to probe a library of Caulobacter strains bearing fluorescent fusions to 309 different localized proteins. From this screen, we identified six proteins that were enriched in the constrictions, three of which are previously uncharacterized and highly conserved in α-proteobacteria (FzlA, FzlB, and FzlC). We demonstrated that three of the six (MurG, FzlA, and FzlC) bind to FtsZ polymers in vitro, doubling the number of known proteins with this activity in Caulobacter and validating our screening approach. Functional analysis of FzlA indicated that it is essential for cytokinesis. Genetic depletion of this factor caused elongation of cells into smooth filaments, reflecting an early role in cytokinesis consistent with its direct interaction with FtsZ. Correspondingly, we found that FzlA mediated the formation of higher order FtsZ structures in vitro and altered FtsZ localization and organization in vivo when overexpressed or depleted. Though FzlC was not essential for viability, its overexpression also caused aberrant cytokinesis and mislocalization of FtsZ. Current studies are focused on dissecting the details of the interactions of FtsZ with FzlA and FzlC to understand how these new interaction partners regulate FtsZ function during cytokinesis.

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ACF7 Regulates Cytoskeletal-Focal Adhesion Dynamics and Migration and Has Intrinsic ATPase Activity.

X. Wu; Rockefeller University, New York, NY

Coordinated interactions between microtubule (MT) and actin cytoskeletons are involved in many polarized cellular processes. Spectraplakins are enormous (>500kDa) proteins able to bind both MTs and actin filaments (F-actin) directly. To elucidate the physiological significance and functions of mammalian spectraplakin ACF7, we've conditionally targeted it in skin epidermis. Intriguingly, ACF7 deficiency compromises the targeting of microtubules along F-actin to focal adhesions (FAs), stabilizes FA-actin networks and impairs

epidermal migration. Exploring underlying mechanisms, we show that ACF7's binding domains for F-actin, MTs and MT plus end proteins are not sufficient to rescue the defects in FA-cytoskeletal dynamics and migration functions of ACF7 null keratinocytes. We've uncovered an intrinsic actin-regulated ATPase domain in ACF7 and demonstrate that it is both functional and essential for these roles. Our findings provide new insights and functions for this important cytoskeletal crosslinking protein in regulating dynamic interactions between MTs and F-actin to sustain directional cell movement.

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A Mechanism for Actin Stress Fiber Homeostasis.

M. Smith[1], E. Blankman[1], M. Gardel[3], L. Luettjohann[1], C. Waterman[2], M. Beckerle[1]; [1]Huntsman Cancer Center, University of Utah, Salt Lake City, UT, [2]Laboratory of Cell and Tissue Morphodynamics, National Institutes of Health, Bethesda, MD, [3]Department of Physics, University of Chicago, Chicago, IL

To maintain mechanical homeostasis of tissues, cells must recognize and respond to changes in cytoskeletal integrity. The actin cytoskeleton, which maintains essential morphological and biophysical features of cells, is subject to mechanical perturbation, but mechanisms to sense and repair local cytoskeletal damage have not been identified. By imaging live cells expressing fluorescently tagged actin, we observed localized sites along actin stress fibers (SF) undergoing cycles of elongation and thinning, followed by repair. Only 17% of localized SF elongation and thinning events progressed to catastrophic breaks. The LIM domain scaffolding protein, zyxin, accumulated rapidly at sites of SF elongation and thinning. In cells generated from zyxin -/- mice, most SF elongation events failed to recover, and the frequency of catastrophic SF breaks increased five-fold. Localized zyxin accumulation on SF could be triggered by direct mechanical perturbation of the cell cortex, indicating these events were force dependent. Furthermore, using traction force microscopy, we showed that stress fiber elongation compromised the ability of SF to transmit stress through focal adhesions to the ECM, and SF repair restored this capability. Along with zyxin, we observed that the actin bundling protein, α-actinin, and the actin capping inhibitor, VASP, accumulated in a zyxin-dependent manner to sites of SF elongation. Thus, we conclude that SF elongation and thinning sites recruit a zyxin-dependent repair complex that serves as a homeostatic system for maintaining SF integrity in the face of mechanical stress.

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Actin-driven Chromosomal Motility Leading to Symmetry Breaking in Mammalian Meiotic Oocytes.

H. Li, R. Li; Stowers Institute for Medical Research, Kansas City, MO

Oocyte meiotic divisions are highly asymmetric, and symmetry breaking initiates with chromosome movement from the oocyte center toward the cortex in many vertebrate and invertebrate organisms. In contrast to invertebrate organism where this movement is microtubule dependent, chromosomal translocation to the cortex in mammalian oocytes is known to be F-actin dependent. Through a combination of 2-photon imaging, 4D tracking, and genetic and pharmacological manipulations, we show that chromosome movement to the cortex is driven by actin polymerization, but not through myosin-based systems. Using a fluorescent peptide that binds specifically to F-actin, we observed a cloud of actin filaments trailing behind the chromosomes/spindle during the movement. Formation of these actin filaments depends on the actin nucleation activity of Fmn2, a formin family protein, which localizes to the chromosome vicinity through its N-terminal region. Symmetry breaking of the actin cloud relative to chromosomes and net chromosome translocation toward the cortex require F-actin turnover. Taken together, our data suggests that meiotic chromosomes are another natural cargo for an actin polymerization-driven motility system. More remarkably, symmetry breaking in this system to achieve an overall displacement is also the origin of symmetry breaking at the cellular level, which enables oocytes to establish cortical polarity and undergo asymmetric meiotic cell divisions.

Minisymposium 7: Lipid Dynamics (46-51)

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Caveolin-1 Domains and the Regulation of Raft-dependent Endocytosis.

P. Lajoie[1], Y. Zheng[2], S. Nim[4], L. Li[1], J. W. Dennis[3], L. J. Foster[2], I. R. Nabi[1]; [1]Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada, [2]Biochemistry, University of British Columbia, Vancouver, BC, Canada, [3]Medical Genetics, University of Toronto, Toronto, ON, Canada, [4]Pathologie et biologie cellulaire, University of Montreal, Montreal, QC, Canada

Caveolin-1 (Cav1), closely associated with caveolae expression, is also found outside caveolae yet its non-caveolar functions remain poorly understood. In mammary epithelial tumor cells derived from polyomavirus middle T (PyMT) transgenic mice deficient for Golgi N-acetylglucosaminyltransferase V (Mgat5), Cav1 levels are reduced and not associated with caveolae expression. Nevertheless, oligomerized Cav1 microdomains still function to negatively regulate EGFR signaling and the cell surface diffusion of both EGFR and cholera toxin b-subunit (CT-b) (Lajoie et al, J. Cell Biol. 179:341-56, 2007). GM1-bound CT-b enters the cells via clathrin-dependent as well as both dynamin-dependent and dynamin-independent raft endocytic pathways. Cav1 microdomains in Mgat5+/+ or Mgat5-/- cells inhibit the dynamin-dependent, raft-mediated endocytosis of CT-b to the Golgi relative to Mgat5-/-ESC cells that lack Cav1. Cav1 negative regulation of CT-b internalization requires an intact scaffolding domain but not Cav1 phosphorylation. Cholesterol depletion and overexpression of either Cav1 or dynamin mutant K44A block CT-b endocytosis at the cell surface, as measured by flow cytometry. However, only minimal colocalization of CT-b and Cav1 is observed. These results suggest that Cav1, in oligomerized Cav1 microdomains, indirectly regulates the raft-dependent endocytosis of CT-b. Quantitative proteomic analysis of detergent resistant membranes (DRMs) from Mgat5+/+, Mgat5-/- and Mgat5-/-ESC cells validated the differential Cav1 expression between these cell lines and identified multiple proteins to be selectively associated with DRMs in caveolae-expressing Mgat5+/+ cells, including known caveolae/raft-associated proteins such as flotillin, filamin A and multiple G protein subunits. However, Cav1 also functions independently of caveolae to regulate raft-dependent endocytosis indirectly via mechanisms that remain to be defined. Supported by the Canadian Institutes of Health Research.

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Invadopodia Biogenesis and Extracellular Matrix Degradation Are Regulated by Caveolin-mediated Modulation of Plasma Membrane Cholesterol Levels.

G. Caldieri, R. Buccione; Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy

Invadopodia are proteolytically active protrusions formed by invasive tumoral cells grown on an extracellular matrix (ECM) substratum. Signal transduction, cytoskeleton remodeling and protease activities converge on invadopodia to allow tightly regulated focal degradation of the ECM. Invadopodia might thus be specialized membrane domains, acting as sites of local signal transduction and membrane remodeling and polarized delivery of components required for ECM degradation. We investigated the features of invadopodia membranes and how altering their composition would affect invadopodia biogenesis and function. This was achieved through multiple approaches including 1) the verification of the detergent-resistance of invadopodia; 2) analysis of the floatation profile of cortactin and dynamin2, typical invadopodia components, by equilibrium ultracentrifugation from cells with or without active invadopodia; 3) manipulation of cholesterol and sphingolipid levels at the plasma membrane; 4) alteration of cholesterol trafficking by caveolin1 knockdown and expression of caveolin3 DGV, a mutant known to block cholesterol transport to the plasma membrane. We found that invadopodia are resistant to Triton-X 100 extraction, and cortactin and dynamin2 shift to lighter (i.e. cholesterol-rich) fractions when cells form actively degrading invadopodia. Furthermore, direct and indirect cholesterol depletion impairs invadopodia biogenesis, persistence and function. Caveolin1 was found to be fundamental to ensure correct cholesterol homeostasis at the plasma membrane as the inhibitory effects of caveolin1 knock-down and caveolin3 DGV expression are reverted by simple provision of cholesterol. We suggest that invadopodia biogenesis and functionality rely on appropriate levels of plasma membrane cholesterol, and that invadopodia display the properties of cholesterol-rich membranes and platforms for polarized trafficking. Most importantly, caveolin1 appears to exert its function in invadopodia formation by regulating cholesterol balance at the plasma membrane.

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Role of Cell Surface Sialic Acid Residues on Plasma Membrane Domains and Caveolar Endocytosis.

R. D. Singh[1,2], C. L. Wheatley[1,2], E. L. Holicky[1,2], D. L. Marks[1,2], R. E. Pagano[1,2]; [1]Medicine, Mayo Clinic College of Medicine, Rochester, MN, [2]Biochemistry & Molecular Biology, Mayo Clinic, Rochester, MN

Plasma membrane (PM) domains enriched in cholesterol and sphingolipids (SLs) are involved in the regulation of processes such as signal transduction and sorting of endocytic cargo (e.g., SV40 virus, fluorescent lipid analogs, SL binding toxins, and GPI-anchored proteins) for subsequent internalization. In this study we explored the role of endogenous sialic acid containing lipids and proteins on various endocytic mechanisms using enzymatic treatments to cleave cell surface sialic residues on human skin fibroblasts (HSFs). When HSFs were pretreated with neuraminidase (cleaves sialic acid from PM gangliosides and proteins) or endoglycoceramidase (cleaves sialic acid from PM gangliosides), PM domain formation, assessed by monomer/excimer formation of Bodipy-lipids, was inhibited and uptake of a caveolar marker was blocked. Immunofluorescence studies of caveolin-1 (Cav1) using TIRF microscopy indicated that these treatments dramatically reduced Cav1 at the PM of cells. This was further verified by EM studies which showed ~80 % reduction in the number of PM caveolae. In contrast, there was no effect of these treatments on the uptake of endocytic markers for the clathrin, or fluid phase pathways in these experiments. The importance of sialic acid containing gangliosides was further demonstrated by subsequently incubating neuraminidase-treated cells with natural gangliosides. When these experiments were performed with various sialic-acid containing gangliosides, caveolar uptake was restored, while incubation with asialo-gangliosides had little effect on caveolar endocytosis. To complement these studies, we also carried out endocytosis assays in Lec2 cells, a mutant CHO cell line that is deficient in CMP-sialic acid transferase and therefore does not synthesize sialic acid-containing lipids or proteins. These cells showed a significant decrease in uptake via caveolae compared to parental cells, with no effect on the clathrin or fluid phase pathway. Together these results demonstrate the importance of cell surface sialic acid residues in supporting PM domain formation and maintenance of caveolar endocytosis. Supported by NIH Grant GM22942.

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Fungal Particle Recognition by C-type Lectin Domains in Dendritic Cells.

A. Neumann, K. Jacobson; Department of Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Fungal organisms can cause human infections ranging from minor irritations to life-threatening systemic events, especially in immuno-compromised patients. The innate immune response is key to the efficient recognition and eradication of these pathogens with macrophages and dendritic cells (DC) forming a major component of this defense. Yeast cell walls are rich in complex carbohydrate polymers such as mannan and β-glucans, and dendritic cells can sense these pathogen signals via C-type lectin receptors (CLR) involving cooperation between DC-SIGN and Dectin-1. DC-SIGN is thought to be important for capture of fungal pathogens and it spontaneously forms microdomains in the plasma membranes of cell lines and DC. We have found that these domains are diffusively isolated from the surrounding membrane in resting DC. They were insensitive to disruption by F-actin destabilizing drugs, cholesterol depletion and a cytoplasmic domain deletion mutant expressed in NIH/3T3 cells was competent to form membrane microdomains. We found that Dectin-1 expressed in DC is also present in membrane domains, and significant colocalization between DC-SIGN and Dectin-1 domains is observed. Eighteen-hour exposure of DC to zymosan (killed S. cerevisciae) results in a time-dependent global increase of the characteristic domain FWHM diameter for Dectin-1 domains (from 768 to 852 nm) but considerably less overall change for DC-SIGN domains (from 838 to 856 nm) as measured by 2D spatial image correlation analysis. The ratio of DC-SIGN/Dectin-1 within DC-SIGN-positive domains does not exhibit a significant global change after zymosan exposure. Zymosan associated DC-SIGN co-migrates with the particle as it undergoes centripetal transport from the peripheral lamella to the perinuclear zone prior to Dectin-1 dependent and independent forms of phagocytosis. Thus CLR domains are reorganized by DC in response to pathogen exposure and participate in capture, transport and internalization of yeast. This work was supported by NIH GM 41402 and F32 AI71900.

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Cortical Cytoskeletal Structures Dimensionally Constrain CD36 Motion at the Cell Surface to Enhance Its Aggregation and

Signaling Function.

K. Jaqaman[1], H. Kuwata[2], N. Touret[3], W. Trimble[2], G. Danuser[1], S. Grinstein[2]; [1]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]Department of Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada, [3]Department of Biochemistry, University of Alberta, Edmonton, AB, Canada

Receptor aggregation in response to ligand binding is prerequisite to signal transduction in many pathways. CD36 is a key receptor in human macrophages, binding to multivalent ligands such as oxidized LDL and malaria-infected red blood cells. To study the dynamics and aggregation kinetics of CD36 receptors in the plasma membrane, we used single-molecule imaging combined with single-particle tracking and mathematical modeling of individual receptor behavior. We immuno-labeled CD36 receptors with a primary Fab fragment and a Cy3-conjugated secondary Fab fragment or with quantum dots, and imaged the dorsal surface of macrophages using live-cell epifluorescence microscopy. To track the imaged CD36 receptors, we developed a single-particle tracking algorithm that detects particles with sub-pixel localization and follows dense particle fields, capturing particle association and dissociation events and recovering trajectory interruptions resulting from temporary particle disappearance (Jaqaman et al. Nat. Methods 2008). We found that, at rest, surface-bound CD36 receptors existed as discrete multimers that contained up to six monomers. A subpopulation of the detected CD36 multimers (~30%) moved along linear tracks that radiated from the perinuclear region. This linear movement required both the acto-myosin network and microtubules. Temporal multi-scale analysis of receptor frame-to-frame displacement and run-time toward and away from the perinuclear region using sampling frequencies of 10-125 Hz revealed that the linear motion of CD36 multimers was due to constrained diffusion within cytoskeleton-mediated linear troughs in the membrane. Importantly, the dimensionality reduction resulting from motion within these troughs increased the probability of CD36 aggregation two-fold. Cytoskeleton perturbations that inhibited the linear motion of CD36 inhibited the uptake of its ligands and the phosphorylation resulting from CD36 cross-linking. These data provide the first direct demonstration of the functional requirements of cortical cytoskeletal structures in the regulation of receptor activation via the spatial organization of receptor motion and aggregation at the level of the plasma membrane.

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Details of Lipid Membrane Dynamics Revealed by Nanoscale Far-Field Fluorescence STED Microscopy.

C. Eggeling[1], C. Ringemann[1], R. Medda[1], G. Schwarzmann[2], K. Sandhoff[2], S. W. Hell[1]; [1]NanoBiophotonics, Max-Planck Institute for Biophysical Chemistry, Goettingen, Germany, [2]Kekulé-Institute for Organic Chemistry and Biochemistry, Bonn, Germany

A whole range of membrane-associated processes are considered to be mediated through cholesterol-assisted lipid interactions such as the integration into lipid nanodomains ('rafts'). However, controversy on their existence still remains, mostly due the lack of suitable detection techniques in living cells. The limited resolution of >200nm of conventional (non-invasive) far-field fluorescence microscopy precludes the direct observation of such lipid dynamics, which are believed to occur on smaller spatial scales. Further, the rapid diffusion also prevents the fast tracking of single lipid molecules in their native cargo-free state. We report the direct and non-invasive detection of single diffusing lipid molecules in nanosized areas in the plasma membrane of living cells using the superior spatial resolution of stimulated emission depletion (STED) far-field microscopy. Combining a (tunable) resolution of down to 30 nm with tools such as fluorescence correlation spectroscopy (FCS), we directly reveal marked differences between lipid classes. Unlike phosphoglycerolipids, sphingolipids are transiently (~ 10 ms) trapped on the nanoscale in cholesterol-mediated molecular complexes. The detection of molecular dynamics in nanoscale areas of tunable size constitutes a powerful new approach to study the dynamics of biomolecules in living cells.

Minisymposium 8: Single Molecule Studies (52-57)

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Microtubule Plus-end Tracking by CLIP-170 Requires EB1.

R. Dixit[1], B. S. Barnett[2], J. E. Lazarus[2], M. Tokito[2], Y. E. Goldman[2], E. Holzbaur[2]; [1]Biology, Washington University, St. Louis, MO, [2]Physiology and Pennsylvania Muscle Institute, University of Pennsylvania, Philadelphia, PA

Microtubules are polarized polymers of tubulin subunits that are essential for a wide range of processes in the cell. Microtubules are inherently dynamic and they alternate between phases of elongation and shortening, particularly at the more dynamic plus-end. A specialized class of microtubule associated proteins, called microtubule plus-end tracking proteins (+TIPs), bind to and specifically track with growing microtubule plus-ends in the cell. The +TIP family consists of numerous structurally unrelated proteins that regulate microtubule dynamics and mediate their interactions with other cellular components. To dissect the molecular mechanisms responsible for +TIP tracking activity, we reconstituted the +TIP tracking of mammalian proteins EB1 and CLIP-170 in vitro at single molecule resolution using time-lapse total internal reflection fluorescence microscopy. We show that EB1 is intrinsically capable of tracking growing microtubule plus-ends over a broad range of concentrations. Single molecule studies demonstrate that EB1 rapidly exchanges at microtubule plus-ends with a dwell time of < 1 s, indicating that single EB1 molecules exhibit multiple rounds of binding and dissociation during microtubule polymerization. In the absence of EB1, CLIP-170 nonspecifically binds along the length of microtubules and exhibits one-dimensional lattice diffusion. We show that the addition of EB1 is both necessary and sufficient to mediate plus-end tracking by CLIP-170. Single molecule analysis of the CLIP-170-EB1 complex also indicates a short dwell time at growing plus-ends, an observation that is inconsistent with a copolymerization model for plus-end specific localization. In addition, when tubulin was polymerized in the presence of the slowly hydrolyzing GTP analog, GMPCPP, plus-end specificity of the CLIP-170-EB1 complex was lost; indicating that the specificity of +TIP tracking is dependent on GTP hydrolysis. Together, these data provide new insight into the mechanisms driving plus-end tracking by mammalian +TIPs, and indicate that EB1 specifically recognizes the distinct lattice structure at the growing microtubule end. This work was supported by grants GM068591 and AR051174.

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Dynactin Increases the Processivity of Single Dynein Motors without Microtubule Tethering.

J. Kardon[1], S. L. Reck-Peterson[2], R. D. Vale[1]; [1]Department of Cellular & Molecular Pharmacology and Howard Hughes Medical Institute, UCSF, San Francisco, CA, [2]Cell Biology, Harvard Medical School, Boston, MA

The dynactin complex is required for intracellular transport by dynein, but its cellular functions and mechanism of action are not clear. Mechanistic studies of dynactin have been hindered by the lack of a genetically tractable source of this large complex, which would allow specific labeling and mutation of its components. We have developed a single molecule assay to directly observe the motility of recombinant *S. cerevisiae* dynein-dynactin complexes. We show that dynactin increases the run length of dynein motors, conclusively demonstrating that dynactin is a dynein processivity factor. Prior indirect observations of mammalian dynein suggested that dynactin might increase dynein's processivity by tethering the motor to the microtubule (MT) through its own MT binding domains. We find that the microtubule binding domains of Nip100 (the *S. cerevisiae* $p150^{Glued}$ homologue) are not required for dynactin activity *in vitro*. Photobleaching studies demonstrate that dynein and dynactin function at a 1:1 stoichiometry, additionally ruling out multimerization as the mechanism for dyenin processivity enhancement. However, a segment of Nip100's coiled-coil that interacts with the dynein intermediate chain (CC1) is required for dynein run length enhancement. These results suggest that dynactin increases dynein processivity by allosterically modulating the behavior of the dynein motor, rather than by acting as a microtubule tether. Dynactin function during nuclear segregation *in vivo* similarly requires Nip100 CC1, but not Nip100 MT binding domains, suggesting that dynactin modulation of single molecule dynein motility may be crucial to *in vivo* function. Future studies will use high precision measurements of dynein stepping behavior to address how dynactin modulates the processive motility of dynein motors.

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Single Molecule and Computational Analyses of the Stability and Rigidity of ER/K α-helices Present in Naturally Occurring Proteins.

S. Sivaramakrishnan, J. Spudich; Department of Biochemistry, Stanford University School of Medicine, Stanford, CA

Protein α-helices are ubiquitous secondary structural elements, seldom considered to be stable without tertiary contacts. However, amino acid sequences in proteins that are based on alternating repeats of four glutamic acid (E) residues and four positively charged residues, a combination of arginine (R) and lysine (K), have been shown to form stable α-helices in a few proteins, in the absence of tertiary interactions. We recently reported that this ER/K motif is more prevalent than previously thought, being represented in proteins of diverse function from archaea to humans with a mean α-helical length of ~ 10 nm (S. Sivaramakrishnan, B. J. Spink, A. Y. L. Sim, S. Doniach, J. A. Spudich, Proc. Natl. Acad. Sci. USA, in press). We also used small angle X-ray scattering (SAXS) to show that ER/K α-helices form isolated and extended single α-helices in solution. This is confirmed by single molecule optical trap analyses of the ER/K α-helix in the medial tail domain of myosin VI, which resists bending forces during the swing of the myosin VI lever arm. We extend these studies to ER/K α-helices of varying length (5-30 nm) present in diverse proteins. Molecular dynamics simulations of the folding and side chain interactions in ER/K α-helices show a dynamic pattern of E and R/K side chain interactions that extends along the α-helical backbone and stabilizes the ER/K α-helix. A simple theoretical model based on side chain interactions, suggests a persistence length of ~ 40 nm for the ER/K α-helices, which is consistent with our single molecule analyses. Thus, the ER/K α-helix is an isolated secondary structural element that can efficiently span long distances in proteins, making it a promising tool in designing synthetic proteins. We propose that the significant rigidity of the ER/K α-helix can help regulate protein function, as a force transducer between protein subdomains. We are developing a comparative framework for the stability and rigidity of α-helices, which is fundamental to our understanding of protein structure and function.

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Dynamics of Eukaryotic Transcription through a Nucleosome.

C. Hodges[1], L. Bintu[2], L. Lubkowska[3], M. Kashlev[3], C. Bustamante[1,2,4]; [1]Biophysics Graduate Group, University of California, Berkeley, CA, [2]Department of Physics, University of California, Berkeley, CA, [3]NCI Center for Cancer Research, National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, MD, [4]Howard Hughes Medical Institute, Departments of Chemistry and Molecular & Cell Biology, University of California, Berkeley, CA

During eukaryotic transcription, RNA polymerases frequently encounter nucleosomes as they translocate along chromosomal DNA. Nucleosomes have been implicated in mediating regulatory pauses during elongation and are known to impede transcription *in vitro*, yet many questions remain regarding the mechanics and kinetics of nucleosomal transcription. We have used optical tweezers to follow individual RNA polymerase II (pol II) elongation complexes as they transcribe a DNA template wrapped in a nucleosome. Our experiments show that a nucleosome acts as a dynamic mechanical barrier that increases local pause density, slows pause recovery, and increases the arrest probability of pol II. Our results are in quantitative agreement with a diffusive model of polymerase backtracking where nucleosomal fluctuations slow recovery from backtrack pauses. During elongation, the nucleosomal barrier induces backtrack pauses by obstructing access to downstream DNA. We have concluded that pol II does not actively separate downstream DNA from histones; it instead rectifies unwrapping fluctuations to gain access to nucleosomal DNA. In addition, we have developed a method to follow single nucleosomes immediately after monitoring their transcription, and we find that the process of histone transfer is extremely sensitive to tension in the DNA upstream of the polymerase. This observation is consistent with a model of histone transfer that requires formation of a transient loop between downstream histones and upstream DNA. The work presented here represents the first direct real-time observations of nucleosomal transcription at the single-molecule level, and allows for many future experiments with transcription and nucleosome remodeling factors.

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Peptide "Sled" Carries Adenovirus Proteinase Along Viral DNA to Locate Its Substrates.

P. C. Blainey[1], W. J. McGrath[2], V. Graziano[2], G. Luo[1], X. S. Xie[1], W. F. Mangel[2]; [1]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA, [2]Biology Department, Brookhaven National Laboratory, Upton, NY

Assembly of many, different viruses utilizes precursor proteins that later must be processed by virus-coded proteinases to render the virus particles infectious. How this occurs inside a nascent virion under conditions where three-dimensional diffusion is severely limited, because the proteinases and precursor proteins are tightly bound to the viral DNA, has been an enigma. A solution to this problem was revealed when

we observed, using total internal reflection fluorescence microscopy, complexes of the adenovirus proteinase (AVP) covalently attached to its peptide cofactor pVIc (GVQSLKRRRCF) exhibiting directionless sliding on viral DNA that could last more than one second and cover more than 20,000 base pairs. The mean diffusion constant was 21.0 x 106 (bp)2/s with the variation among diffusion constants measured for individual AVP-pVIc complexes yielding a standard deviation of 15.6 x 106 (bp)2/s. One-dimensional diffusion during virion maturation provides a means for DNA-bound proteinases to locate and process DNA-bound precursor proteins. In a sliding assay of just AVP, we observed many molecules bound to DNA; however, only a few of the AVP molecules appeared to slide, and those molecules that did slide did not exhibit the rapid sliding activity observed for the covalent AVP-pVIc complex. AVP-pVIc complexes slid because the 11-amino acid peptide pVIc was acting as a "molecular sled." pVIc slid quickly and persistently by itself or carried with it as cargo the products of different genes, e.g. AVP, which by itself bound to DNA but did not slide, or the heterologous molecule Streptavidin, which did not bind to DNA. pVIc's one-dimensional diffusion constant for sliding on DNA, 26.0 x 106 (bp)2/s, was the fastest yet observed. Little or no skipping was observed as the one-dimensional diffusion constants did not increase when the salt concentration was increased 10-fold. Sliding of AVP-pVIc complexes along DNA on a "molecular sled" illustrates a new biochemical mechanism by which a proteinase can locate its substrates and represents a new paradigm for virion maturation.

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Export of Imp α/CAS Complexes and Formation during Nuclear Import.

C. Sun[1], W. Yang[2], S. Musser[1]; [1]Department of Molecular and Cellular Medicine, Texas A&M Health Science Center, College Station, TX, [2]Department of Biological Science, Bowling Green State University, Bowling Green, OH

Nuclear pore complexes (NPCs) mediate the exchange of proteins and RNAs between the cytoplasm and nucleoplasm of eukaryotic cells. The permeability barrier consists of a network of natively unfolded polypeptides called FG-Nups that occlude an ~50 nm diameter pore. In order to transit through the FG-Nup network, large (> ~40 kDa), signal-dependent cargos typically form a complex with a member of the importin β (Imp β) superfamily of transport receptors. For some nuclear import cargos, an accessory transport receptor, importin α (Imp α), is required. Using single molecule fluorescence and single particle tracking techniques, we directly monitor individual cargos transiting through NPCs in permeabilized cells. We recently developed a single molecule fluorescence resonance energy transfer (FRET) approach to monitor the dissociation of Imp α/cargo complexes at the NPC and reported that dissociation of this complex requires CAS, the recycling factor for Imp α (Sun et al. 2008 PNAS 105:8613). We have now developed an Imp α/CAS FRET pair (Kd = 13±1 nM), with which we show that ~68% of Imp α molecules that enter the NPC from the cytoplasmic side (presumably as an Imp β/Imp α/cargo complex) bind to a CAS molecule. These data demonstrate that CAS directly interacts with Imp α near the termination of the import process. Surprisingly, ~22% of newly formed Imp α/CAS complexes dissociate at the NPC before release from the pore, suggesting that at least sometimes CAS interacts with Imp α without dissociating the bound cargo. We have also examined export of Imp α by CAS and find that export is biased over return to the nucleoplasm by ~2:1. This bias holds for Imp α/CAS complexes that clearly remain intact upon cytoplasmic or nucleoplasmic release, and thus apparently unchanged during their interaction with the NPC. Thus, the NPC appears able to bias diffusion. This ~2:1 transport bias is also observed for dextran import and export, indicating that it is not the CAS transport cofactor that introduces the bias. We conclude that the FG-Nup network itself can actively promote signal-independent and signal-dependent transport.

E. B. Wilson Medal Presentation and Address (58-59)

58

Proteins That Emit Infrared Fluorescence or Singlet Oxygen.

R. Y. Tsien[1,2], X. Shu[1,2], A. Royant[2]; [1]Howard Hughes Medical Institute, University of California, San Diego, La Jolla, CA, [2]Pharmacology, University of California, San Diego, La Jolla, CA

Infrared fluorescent proteins (IFPs) would facilitate fluorescence imaging in intact animals because absorption from tissue hemes drops steeply beyond 600 nm. IFPs would also benefit microscopic imaging by reducing the contribution of cellular autofluorescence, enabling excitation by cheap laser diodes, adding new wavelengths for multicolor labeling, and accepting fluorescence resonance energy transfer from existing FPs. However, published FPs with inbuilt chromophores, i.e. FPs from jellyfish or corals, have not yet exceeded 595/655 nm in excitation/emission maxima respectively. We now show that phytochromes incorporating tetrapyrrole chromophores can be mutated into monomeric IFPs, with typical excitation/emission maxima at 686/713 nm respectively, extinction coefficients >90,000 $M^{-1}cm^{-1}$, and quantum yield up to 0.08 so far. The IFPs express well in mammalian cells and spontaneously incorporate the chromophore, which can either be supplied exogenously or enzymatically synthesized in situ. Alternatively, the phytochromes can incorporate a different tetrapyrrole, which photogenerates singlet oxygen with a quantum yield of 0.15. Thus a single fusion protein, depending on which chromophore is loaded and what excitation wavelength is applied, should emit either infrared fluorescence or singlet oxygen. The latter enables electron microscopic imaging and chromophore-assisted light inactivation.

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GFP: Shedding Light on Biological Processes.

M. Chalfie; Department of Biological Sciences, Columbia University, New York, NY

Since its introduction as a biological marker, the *Aequorea victoria* green fluorescent protein (GFP) has had a strong impact in biology, being used in an ever increasing variety of ways. The ability to have a genetically-encoded marker that can be visualized in living tissues and does not require additional cofactors has certainly changed the way we do research on *Caenorhabditis elegans* in my laboratory. We have used GFP in promoter and protein fusions 1) to characterize spatial and temporal gene expression patterns; 2) to characterize the subcellular distribution of proteins; 3) to identify mutations affecting specific cells or processes; 4) to label cells so they can be identified in culture or isolated by fluorescence-activated cell sorting; 5) to label cells for electrophysiological characterization; and 6) to develop additional biological methods.

Golgi Complex (61-76)

61/B2

A Nitric Oxide (NO) Scavenger, carboxy-PTIO, Fragmented the Golgi Apparatus in Endothelial Cells.

J. Lee, P. Sehgal; Cell Biology, New York Medical College, Valhalla, NY

S-nitrosylation is emerging as an important bidirectional regulator of protein function - as examples the negative regulation of eNOS and positive regulation of ras activities. In evaluating the effect of an NO scavenger, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO) on bovine pulmonary arterial endothelial cells (PAECs) in culture we discovered that cPTIO markedly fragmented the Golgi apparatus and moved a subset of these cells into mitosis. The compact juxtanuclear Golgi in PAECs was extensively fragmented by 20-24 hr of exposure to cPTIO (typically at 100 µM) as assayed in terms of immunostaining for the Golgi scaffolding proteins giantin or GM130 or the trans-Golgi SNARE syntaxin 6. This fragmentation, which was reversible upon washout of cPTIO, coincided with reduced cellular NO levels as assayed in live-cells using DAF2-DA immunofluorescence, reduced localization of BODIPY-C5-ceramide in the Golgi elements and hypo-S-nitrosylation of eNOS, caveolin-1 and clathrin heavy chain. Despite Golgi fragmentation, soluble cargo such as horse-radish peroxidase, produced off an exogenously transfected vector, was secreted in the continued presence of cPTIO for at least 6 days. These data suggest that NO, and thus S-nitrosylation, is required for the structural integrity of the Golgi apparatus in endothelial cells. The specific protein target(s) remains to be identified. Supported by HL-073301 and HL-087176.

62/B3

RhoBTB3: A Rho GTPase-family ATPase Required for Endosome to Golgi Transport.

E. Espinosa, M. Calero, K. Sridevi, S. Pfeffer; Biochemistry, Stanford University, Stanford, CA

Mannose 6-phosphate receptors deliver newly synthesized lysosomal enzymes to endosomes and then recycle back to the Golgi. Retrograde transport from late endosomes to the trans Golgi network requires the small GTPases Arl1, Rab6 and Rab9, TIP47, GCC185, a SNARE complex comprising STX10, STX16, Vti1a, and VAMP3, as well as cytoplasmic dynein and intact microtubules. We report here that the atypical Rho GTPase family member, RhoBTB3, is a Rab9 effector that is also required for endosome to Golgi transport. RhoBTB3 resides on the trans Golgi network and cells depleted of this protein mislocalize mannose 6-phosphate receptors and TGN46 into small peripheral vesicles, display an expanded (but intact) trans Golgi network, and hyper-secrete lysosomal enzymes. Mutagenesis of RhoBTB3 permitted identification of the precise Rab9 binding site, and yielded an additional mutant protein that does not bind nucleotide. Gene replacement experiments show that RhoBTB3 function in cultured cells requires both RhoBTB3's N-terminal, Rho-related domain--specifically, its nucleotide binding capacity, as well as C-terminal sequences that are important for Rab9 interaction. Surprisingly, biochemical analysis of RhoBTB3's Rho GTPase-related domain reveal that it binds and hydrolyzes ATP rather than GTP, with kinetic properties that resemble small GTPases rather than most ATPases. Together, these data suggest that the RhoBTB3 ATPase functions with both Rab and Arl-family GTPases in vesicle docking at the trans Golgi network.

63/B4

Clavesin 1 and 2, Novel Regulators of Clathrin-mediated Traffic from the trans-Golgi Network in Neurons.

Y. Katoh, B. Ritter, F. Blondeau, P. S. McPherson; Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, QC, Canada

We have recently undertaken a subcellular proteomics approach that identified eight novel proteins associated with brain clathrin-coated vesicles (CCVs). Subsequent studies have linked six of these proteins directly to clathrin-mediated trafficking. Of the two remaining proteins, one contains a Sec14 domain, a phospholipid-binding module and we have named it clavesin (clathrin vesicle-associated Sec14 protein) 1. A bioinformatic analysis identified a clavesin 1 homologue (clavesin 2) with a similar domain structure and 79% overall identity. An antibody against a C-terminal peptide from clavesin 1, which reacts with both clavesin isoforms reveals that the proteins are expressed exclusively in brain and are highly enriched on purified CCVs. Immunofluorescence analysis in cultured hippocampal neurons, coupled with lentiviral-mediated clavesin 1/2 knock down demonstrates that clavesins co-localize with clathrin-heavy chain (CHC) and the clathrin adaptor AP-1 on clathrin-coated structures at the *trans*-Golgi network (TGN). Interestingly, both clavesin 1 and 2 bind to AP-1 and CHC through a C-terminal region downstream of the Sec14 domain and to PtdIns(3,4)P_2 through their Sec14 domains. Moreover, the Sec14 domain is sufficient to target either clavesin isoform to TGN membranes when transfected in cells. These data indicate that clavesins function in clathrin-mediated trafficking at the TGN specifically in neurons.

64/B5

Inhibition of Intra-Golgi Retrograde Trafficking (Through Rab33b Depletion) Has Little Effect on Anterograde Traffic or HeLa Cell Multiplication.

T. Starr, Y. Sun, N. Wilkins, B. Storrie; Physiology and Biophysics, University of Arkansas for Medical Sciences, Little Rock, AR

The medial Golgi protein Rab33b is an important regulator of retrograde trafficking through the Golgi apparatus. Hence, we reasoned that knockdown of Rab33b could provide an experimental test of the interrelationship between intra-Golgi retrograde and anterograde trafficking and cell growth and division in mammalian cells. We report that Rab33b knockdown produced if anything a more continuous Golgi ribbon and suppressed COG- or ZW10-depletion induced disruption of the Golgi ribbon in HeLa cells. Both COG and ZW10 have been implicated as tether proteins in Golgi retrograde trafficking. Efficient Rab6 induced relocation of Golgi enzymes to the ER was Rab33b-dependent as was the transport of the Shiga toxin B subunit from the trans Golgi to the ER. Strikingly, however, cell growth and multiplication was normal with Rab33b knockdown. Moreover, transport of VSV-G protein from the ER to plasma membrane via the Golgi apparatus was normal. We conclude that significant uncoupling of Golgi retrograde and anterograde trafficking can occur in mammalian cells with little consequence to cell growth. If, indeed, cisternal maturation is the conveyor by which cargo transport through the Golgi occurs, then there must be multiple mechanisms for supporting the required protein recycling. (Supported by a grant from the NSF.)

65/B6

AGPAT3, a Lysophosphatidic Acid Acyltransferase That Mediates the Functional Organization of the Golgi Complex.

J. Schmidt, W. Brown; Molec. Biol. and Genetics, Cornell University, Ithaca, NY

Recent studies have suggested that the functional organization of the Golgi complex is dependent on phospholipid remodeling enzymes. Here we report the identification of a specific integral membrane AGPAT (AGPAT3/LPATgamma) that regulates Golgi membrane tubule formation, trafficking, and structure. Endogenous and GFP labeled AGPAT3 were localized primarily to the Golgi complex and also to ER membranes. Golgi membranes isolated from AGPAT3 over-expressing cells had elevated lysophosphatidic acid (LPA) acyltransferase activity, and mutations in the catalytic site abolished this activity. Interestingly, over-expression of AGPAT3 significantly inhibited the formation of Golgi membrane tubules in vivo by brefeldin A (BFA) and in vitro using a cytosol-dependent reconstitution system. These tubules were shorter, fewer in number, and significantly slowed retrograde traffic to the ER. Catalytically inactive AGPAT3 did not show this phenotype. In addition to membrane tubule disruption, both anterograde and retrograde protein trafficking was slower in cells over-expressing AGPAT3 and accelerated in cells with reduced expression (by siRNA). Cells with knocked-down expression of AGPAT3 also displayed highly fragmented Golgi mini-stacks that were hyper-sensitive to BFA. Therefore, AGPAT3 appears to utilize acyl-CoA and LPA to generate phosphatidic acid in Golgi membranes that contribute to maintaining Golgi structure and/or the formation of tubules and vesicles for the intracellular transport of proteins and lipids. These data are the first to show a direct role for a transmembrane phospholipid acyltransferase in regulating membrane trafficking and organelle structure.

66/B7

Multiple Rab GTPase Binding Sites in GCC185 Suggest a Model for Vesicle Tethering at the Trans Golgi Network.

G. Hayes, F. C. Brown, R. Nottingham, S. Pfeffer; Biochemistry, Stanford University, Stanford, CA

GCC185 is a trans Golgi network-localized protein predicted to assume a long, coiled coil structure that is required for Rab9-dependent recycling of mannose 6-phosphate receptors (MPRs) to the Golgi as well as for microtubule nucleation at the Golgi via CLASP proteins. GCC185 achieves its Golgi localization by cooperative interaction with Rab6 and Arl1 GTPases at adjacent sites near its C-terminus. We have utilized a yeast two hybrid screen to identify at least four additional binding sites for as many as 14 different Rab GTPases across its entire length. A central coiled coil domain contains a specific binding site for Rab9; functional assays indicate that this domain is important for MPR recycling to the Golgi complex. N-terminal coiled coils are also required for GCC185 function in Golgi structural organization as determined by plasmid rescue after GCC185 depletion using siRNA in cultured cells. Finally, If GCC185 is a fully extended coiled-coil protein, it is predicted to reach 240nm in length from the trans Golgi network. We tested whether GCC185 could reach from the trans Golgi to the cis Golgi using N- and C-terminal specific tags. In our immuno-fluorescence experiments, we did not detect significant co-localization of the GCC185 N-terminus with cis Golgi markers, GM130 or p115. Thus, it appears that GCC185 does not project in this direction as far as the cis Golgi; it may be bent or instead, extending uniquely away from the Golgi stack. Multiple Golgi-Rab binding sites may permit GCC185 to contribute to stacking and lateral interactions of Golgi cisternae as well as to facilitate its function as a transport vesicle tether and microtubule anchoring site.

67/B8

Protein Kinase D2 Recruitment to the trans-Golgi Network Requires the First and Second Cysteine-rich Zinc Finger Domains.

G. Pusapati, G. Adler, T. Seufferlein; Internal Medicine I, University of Ulm, Ulm, Germany

Protein Kinase D2 (PKD2) is a member of the PKD family of serine / threonine kinases, which in turn belongs to the calcium / calmodulin - dependent protein kinase (CAMK) family. PKDs are activated either directly via phorbol esters or indirectly by various mechanisms including G protein-coupled receptors. These kinases are implicated in various biological processes such as proliferation, apoptosis and regulation of Golgi function. PKDs have an N-terminal regulatory domain and a C-terminal kinase domain. The N-terminal regulatory domain comprises two cysteine-rich zinc finger domains (C1a and C1b) and a pleckstrin homology domain. These individual domains regulate the localization and function of PKDs. So far, most data have been obtained examining the regulatory domain of PKD1. Despite striking similarities, there are also distinct differences in the regulatory domains of PKD1, 2 and 3.In the present study we examined the role of the regulatory domain of PKD2 for its precise subcellular localization using mutants. It has been demonstrated that PKD1 is recruited to the *trans*-Golgi network (TGN) via C1a domain. Our data show, that PKD2 in addition to the C1a domain, also needs its C1b domain to localize to the TGN. In contrast to a C1a deletion mutant, PKD2 lacking its C1b domain (PKD2-ΔC1b) is catalytically less active implicating that the C1b domain plays a positive regulatory role. However, the mere decrease in catalytic activity is not responsible for the impaired localization of PKD2-ΔC1b to the TGN as demonstrated by a constitutively active C1b deletion mutant of PKD2, which also does not localize to the TGN.Upon Brefeldin A (BFA) treatment of cells, which is known to cause the dissociation of a number of peripheral Golgi-associated proteins, we observed that wild-type GFP-tagged PKD2 relocalizes into tubular structures. However, upon BFA treatment there is no relocalization into tubular structures either of, GFP-PKD2-ΔC1b or of constitutively active GFP-PKD2-ΔC1b expressing cells. Collectively, these results demonstrate a major role of the C1b domain in PKD2 for its localization to the TGN and point out clear differences in the functional properties of the regulatory domains of PKD isoforms.

68/B9

A Role for the Acidic Domain of the Golgin Tether p115 in Exocytic Transport.

A. Satoh[1], G. Warren[2]; [1]Cell Biology, Yale University School of Medicine, New Haven, CT, [2]Max F. Perutz Laboratories, Wien, Austria

Golgins are coiled-coil proteins involved in Golgi architecture and function. A complex of golgins (p115, GM130 and giantin), together with the rab1 GTPase and cis-Golgi SNAREs, helps to mediate fusion processes at the entry face of the Golgi apparatus. The C-terminal acidic domain of p115 binds specifically to GM130 and giantin. However, deletion of this domain in vivo appears to have no effect on exocytic transport when using an RNAi depletion/rescue approach (Puthenveedu MA, Linstedt AD. Proceedings of the National Academy of Sciences of the United States of America 2004;101(5):1253-1256). Here we have used a different approach, introducing a tobacco etch virus (tev)

protease cleavage site into p115 so that the C-terminal domain can be rapidly and specifically released in vivo by micro-injection of the tev protease. The results show clearly that cleavage inhibits exocytic transport to the cell surface.

69/B10

Deletion of Subunits of the COG Complex Causes a Delay in Subtilase Cytotoxin Plasma Membrane-to-Endoplasmic Reticulum Retrograde Trafficking.

R. Smith[1], J. C. Paton[2], V. Lupashin[1]; [1]Physiology and Biophysics, UAMS, Little Rock, AR, [2]School of Molecular and Biomedical Science, University of Adelaide, Adelaide, SA, Australia

Subtilase cytotoxin (SubAB) is an AB5 exotoxin produced by the Shiga Toxogenic Escherichia coli. The five B subunits bind on to glycosylated receptors on the plasma membrane. The toxin displays clathrin-dependent endocytosis and its retrograde traffic is blocked by application of Brefeldin A. The A subunit is a serine protease and remains in the ER lumen to specifically cleave the chaperone GRP78. We have performed siRNA-induced knockdowns of proteins thought to be involved in the retrograde traffic to investigate toxin trafficking in HeLa cells. We found that SubAB trafficking was normal in cells depleted for SNX1 and/or SNX2, i.e toxin delivery was retromer-independent. Knockdown (KD) of either lobe A or lobe B subunits of the COG complex resulted in a significant delay in the cleavage of GRP78 by SubAB. While the deletion of a COG subunit can cause altered glycosylation of toxin receptors, our data indicated that plasma-membrane binding of fluorescently-labeled SubAB was not altered in COG KD cells. Additionally, knockdown of Rab6 and β-COP resulted in similar delays in GRP78 cleavage as in COG subunit knockdowns; knockdown of Syntaxin 5 completely blocked toxin trafficking; while knockdown of p115 did not alter SubAB delivery. Staining Vero cells with fluorescently-labeled SubAB demonstrated that SubAB was localized to the perinuclear area 20 min after intoxication, while in the COG3 KD Vero cells the SubAB was peripheral and not co-localized to Giantin-positive cid-Golgi structures. Knockdown of COG subunits and other trafficking factors will allow us to better understand the retrograde trafficking of bacterial toxins through the Golgi apparatus

70/B11

The COG Complex Is Separated into Two Sub-complexes on the Membrane to Direct Trafficking of Golgi Enzymes.

R. Smith[1], R. A. Willett[1], W. Morelle[2], V. Lupashin[1]; [1]Physiology and Biophysics, UAMS, Little Rock, AR, [2]Laboratory of Structural and Functional Glycobiology, University of Lille, Lille, France

The Conserved Oligomeric Golgi (COG) complex is a vesicle tethering factor for retrograde trafficking within the Golgi apparatus. The peripheral membrane COG complex is composed of lobe A (COG1-4) and lobe B (COG5-8). Mutations of Cog1, Cog7, and Cog8 subunits cause a type II human congenital disorder of glycosylation (CDG-II). We have performed differential centrifugation and co-immunoprecipitations of different COG subunits from HeLa cells to test the existence of several membrane-bound COG complexes and to determine COG subunit(s) responsible for membrane attachment. Using a shRNA plasmid and siRNA duplexes we have knockdown (KD) COGs2, 3, 4, 6 and 8. Our results demonstrated that COG subcomplexes Lobe A and Lobe B are, at least partially, temporarily and spatially separated on Golgi membranes and that several subunits from both lobes are responsible for membrane attachment. Brefeldin A-induced relocalization of Golgi resident proteins to ER was found to be significantly delayed in a COG subunit depleted cells compared to control cells. All COG subunits KDs in lobe A and lobe B exhibit an increase in GS-II (specifically binds to terminal N-acetylglucosamine) and Galanthis nivalis (specifically binds to terminal mannose) lectins staining compared to control cells, indicated hyposialylation, hypogalactosylation, and alteration of N-acetylglucosamine transferase. Furthermore, MALDI-TOF analysis of glycoproteins from cells deficient in COG complex has shown a decrease percent intensity of N-linked sugars corresponding to the increase in lectin-binding. By using KDs of COG subunits, it will allow us to understand retrograde trafficking in the Golgi and the segregation of glycosyltransferases and glycosidases in different Golgi cisternae.

71/B12

Expression and Subcellular Localization of Diacylglycerol Kinase Isozyme in Rat Aortic Endothelial Cells.

T. Nakano[1], K. Goto[2], I. Wakabayashi[1]; [1]Environmental and Preventative Medicine, Hyogo College of Medicne, Nishinomiya, Japan, [2]Anatomy and Cell Biology, Yamagata University School of Medicine, Yamagata, Japan

Diacylglycerol kinase (DGK) is an enzyme responsible for phosphorylation of diacylglycerol (DG) to phosphatidic acid (PA). DG is known to activate conventional and novel classes of protein kinase C (PKC), RasGRP and transient receptor potential (TRP) channel. In addition, PA also acts as an activator for atypical PKC, hypoxia inducible factor-1α and mammalian target of rapamycin, suggesting that DGK plays a pivotal role in a variety of intracellular signaling. Endothelial cells respond to extrinsic stimuli and produce several bioactive substances, such as NO or PGI2. It is known that TRP channels and/or PKCs play important roles in the process of the endothelial signaling. However, little is known about the regulatory mechanism of the intracellular signaling by DGK in endothelial cells. The present study aims to investigate the expression and subcellular localization of DGK isozymes in rat aortic endothelial cells (RAECs). RT-PCR revealed that α, γ, ε and ζ subtypes of DGK isozymes were expressed in RAECs. Immunocytochemical examination demonstrated that DGKα was detected diffusely in the nucleus and cytoplasm while DGKζ localized to the nucleus. DGKγ was detected in the cytoplasm as tubular or reticular structure, which was immunoreactive for Golgin 97, a marker for the Golgi complex. When incubated with brefeldin A (BFA), which is known to reversibly disrupt the Golgi complex, DGKγ adopted a punctate, fragmented morphology. In 30 min after washing out BFA, DGKγ regained a Golgi-pattern distribution. Thus, these results suggest that DGKγ plays some role in the Golgi complex in RAECs.

72/B13

PLD Is Involved in the Formation of Golgi Associated Clathrin Coated Vesicles in HSY Cells.

L. B. Souza, M. Jamur, C. Oliver; Cell and Molecular Biology, FMRP USP, Ribeirão Preto, Brazil

Phospholipase D (PLD) has been implicated in many cellular functions, such as vesicle trafficking, exocytosis, differentiation, and proliferation. The function and intracellular localization of PLD varies according to cell type. The aim of this study was to characterize the role of PLD in HSY cells, a human cell line originating from the intercalated duct of the parotid gland. Treatment of cells with the primary

alcohol 1-butanol inhibits the hydrolysis of phosphatidylcoline by PLD thereby suppressing phosphatidic acid (PA) production. Instead, phosphatidyl-1-butanol is formed by a transphosphatidylation reaction. In untreated HSY cells, there was only a slight co-localization of PLD with the clathrin coated vesicles. PLD was localized to cytoplasmic vesicles and clathrin coated vesicles could be found throughout the cell with an increased concentration in a juxtanuclear region. When HSY cells were incubated with 1-butanol the total number of clathrin coated vesicles increased, especially in the juxtanuclear region and the co-localization of PLD with the clathrin coated vesicles was augmented. Transmission electron microscopy confirmed that the number of Golgi-associated coated vesicles was greater. Treatment with 1-butanol also affected the Golgi apparatus, increasing the volume of the Golgi saccules. The decrease in PA levels after treatment with 1-butanol also resulted in an accumulation of lysosomes in the perinuclear region. Therefore, in HSY cells PLD appears to be involved in the formation of Golgi associated clathrin coated vesicles as well as in the structural maintenance of the Golgi apparatus.

73/B14

CERT Localization and Sphingomyelin Synthesis during Apoptotic Stress and Cell Cycle Events.

S. Chandran, C. Machamer; Cell Biology, Johns Hopkins University Scool of Medicine, Baltimore, MD

The mammalian Golgi apparatus is composed of multiple stacks of cisternal membranes organized laterally into a ribbon-like structure, with close apposition of trans Golgi regions with specialized endoplasmic reticulum (ER) membranes. These contacts may be the site of ceramide transfer from its site of synthesis (ER) to sphingomyelin (SM) synthase via ceramide transfer protein (CERT). CERT extracts ceramide from the ER and transfers it to Golgi membranes. We have previously shown that CERT is enriched at the Golgi region and the Golgi localization of CERT puncta requires both ER and Golgi binding domains of CERT. We examined the contribution of Golgi structure to SM synthesis by treating cells with Golgi-perturbing drugs including ilimaquinone, nocodazole and brefeldin-A. Interestingly, only disruption of Golgi morphology with nocodazole inhibited newly synthesized SM, while the other drugs had no significant effect on SM synthesis. Confocal microscopy showed that decreased SM synthesis in nocodazole-treated cells correlated with decreased localization of CERT to nocodazole-induced Golgi ministacks, indicating that some Golgi structural perturbations interfere with efficient ceramide trafficking through CERT, and thus SM synthesis. Other possibilities including the inhibitory effect of nocodazole on SM synthase activity, alteration of phosphorylation status of CERT and elimination of CERT's association with Golgi membranes by nocodazole were ruled out, suggesting that the organization of the mammalian Golgi ribbon together with CERT may promote specific ER-Golgi interactions for efficient delivery of ceramide for SM synthesis. We are also examining the correlation between CERT localization and SM synthesis during other Golgi perturbations including proapoptotic stress and the cell cycle. Our preliminary results suggest that CERT localization is disrupted during the late G2 phase of the cell cycle, as well as after treatment of cells with proapoptotic stimuli. We are currently analyzing CERT dependent ceramide trafficking during the cell cycle and during proapoptotic stress to gain further insight into the contribution of Golgi structure to SM synthesis.

74/B15

Neogenesis and Maturation of a Naturally Pulsed, Golgi-like Organelle System during Stage-Differentiation in Giardia lamblia.

S. Stefanic, L. Morf, C. Konrad, A. B. Hehl; University of Zurich, Zurich, Switzerland

The protozoan parasite *Giardia lamblia* has a basic but effective trafficking system and lacks a classical Golgi apparatus with stacked cisternae. During evolution Giardia has undergone dramatic reduction of all organelle systems and machineries investigated to date, but still possesses sophisticated secretory and endocytic transport pathways. For transmission to new hosts, trophozoite stages undergo a stage-differentiation process termed encystation which is completed after rapid secretion of a massive extracellular matrix (cyst wall) composed of only three cyst wall proteins (CWPs) and a GalNAc homopolymer. Large amounts of CWP is synthesized for a limited time, exported from the ER and sorted rapidly into 20-30 encystation-specific vesicles (ESVs). Although ESVs have no similarity to the classical Golgi of higher eukaryotes, all available data suggest that ESVs may be unique developmentally regulated Golgi-like organelles which arise de novo during encystation and undergo synchronous cisternal maturation. To test this hypothesis we probed the regulated secretory system of Giardia using conditional expression of dominant-negative variants of small GTPases. The combined data shows that ESV neogenesis follows universally conserved principles and is remarkably similar to ER to Golgi transport despite a completely different morphofunctional organization. Time-lapse microscopy analysis using a CWP1::GFP chimera as a cargo marker demonstrates that ESVs mature into an organelle network reminiscent of the Golgi ribbon. Quantitative analysis reveals rapid exchange of cargo between ESVs via dynamic tubular connections. We also show processing-dependent dense core formation in mature ESVs. This is linked to cargo sorting prior to regulated secretion of the cyst wall material in two stages and key to achieving environmental resistance necessary for transmission to a new host. The naturally pulsed nature of this Golgi-like export system in Giardia provides a unique model to study universally conserved processes in secretory granule formation in a highly simplified context.

75/B16

Myosin-dependent Fission of Rab6-positive Transport Intermediates from the Golgi Complex.

S. Miserey-Lenkei[1], G. Chalancon[1], S. Bardin[1], L. Daviet[2], B. Goud[1], A. Echard[1]; [1]Institut Curie UMR144 CNRS, Paris, France, [2]Hybrigenics, Paris, France

The Rab6 GTPase regulates bidirectional trafficking at the Golgi interface in mammalian cells. Indeed, Rab6 controls both a retrograde transport pathway between endosomes and the endoplasmic reticulum via the Golgi complex and an anterograde pathway between the Trans Golgi Network and the plasma membrane. We previously showed that Rab6-positive transport intermediates (Rab6-ITs) move along the microtubule network in a bidirectional manner, but whether the Rab6 function depends on the actin cytoskeleton is unknown. In order to identify the molecular motors implicated in either the movement and/or the fission of Rab6-ITs, we systematically depleted dynein, kinesins or myosins by RNAi in Drosophila S2 cells and followed the Rab6-GFP dynamics using high-resolution time-lapse microscopy. We identified a myosin whose inhibition affected Rab6-ITs behaviour. Depletion of the corresponding myosin orthologue in human cells led to the formation of long unfissioned Rab6-positive tubes connected to the Golgi apparatus and a concomitant reduction in the number of Rab6-ITs. In those tubes, markers of the Rab6-regulated retrograde and anterograde pathways were present. Interestingly, Rab6 directly interacts

with this myosin and controls its recruitment to the Golgi apparatus. We conclude that actin-based motors are implicated in the fission of Rab6-ITs from the Golgi complex.

76/B17

Characterization of mechanisms that regulate recruitment of BIG1 to TGN membranes.

M. Styers, T. Szul, J. Lowery, E. SZTUL; Cell Biology, University of Alabama at Birmingham, Birmingham, AL

GBF1, BIG1, and BIG2 are members of the Sec7 family of GTP Exchange Factors (GEFs) that catalyze the exchange of GTP for GDP on ADP-Ribosylation Factor (ARF) proteins. Activated ARF is required for coat recruitment and vesicle biogenesis in membrane transport. These three large GEFs are all characterized by sensitivity to the fungal metabolite Brefeldin A (BFA), which stabilizes an abortive tertiary complex between the GEF, ARF, and BFA. Each GEF exhibits a distinct membrane localization. GBF1 is found at Endoplasmic Reticulum-Golgi Intermediate Compartment (ERGIC) and Golgi membranes, while BIG1 and BIG2 have been reported to localize to the Trans-Golgi Network (TGN), and the TGN and recycling endosomes, respectively. To date, the mechanisms that regulate membrane recruitment of GBF1, BIG1, and BIG2 have only partially been characterized. Our laboratory has previously shown that GBF1 recruitment is regulated both by the small GTPase Rab1b and by its substrate, Arf1. However, the mechanisms that regulate BIG1 and BIG2 recruitment to the membrane remain poorly understood. We have found that treatment of cells with BFA leads to stabilization of GBF1 and BIG2 on cellular membranes. However, BFA treatment only partially stabilizes BIG1 on the membrane. These results suggest that activated ARF may play a role in recruitment of BIG1 to TGN membranes. We describe experiments aimed at dissecting the relationship between GEF function, activation of specific ARF proteins, and the recruitment of BIG1 to TGN membranes.

Targeting to Lysosomes (77-86A)

77/B18

Golgi to lysosome transport of lysosomal proteins.

J. Klumperman[1], E. van Meel[1], M. Boonen[2], S. Kornfeld[2]; [1]Department of Cell Biology, University Medical Centre Utrecht, Utrecht, Netherlands, [2]Department of Internal Medicine, Washington University School of Medicine, St. Louis, MO

The biogenesis of lysosomes requires transport of lysosomal hydrolases and lysosomal membrane proteins from their site of synthesis, the endoplasmic reticulum, to lysosomes. The objectives of our studies are to characterize these pathways, especially those that involve direct transport from the trans-Golgi network (TGN) to the endosomal system. Lysosome-associated membrane proteins (LAMPs) travel to the endosomal system via an indirect (passaging the plasma membrane) or direct (entirely intracellular) pathway. The morphological and molecular identity of the membrane carriers and compartments involved in these pathways are largely unknown. In order to study these, we combined RNAi, live cell imaging and immuno-electron microscopy and also performed correlative live cell imaging - electron microscopy (CLEM). Our present data implicate a novel, direct TGN-to-late endosome pathway for LAMP-1. Transport of lysosomal hydrolases from the TGN to the endosomal system is mediated by mannose 6-phosphate receptors (MPRs), through recognition of mannose 6-phosphate (M6P) moieties present on the newly synthesized hydrolases. However, several lines of evidence indicate that lysosomal hydrolases can also be transported via a MPR-independent pathway. For instance in I-cell disease, a disorder characterized by reduced activity of the GlcNAc-1-phosphotransferase (Ptase) resulting in the absence of the M6P modifications, the enzyme content of lysosomes in a number of tissues is near normal. To study the effect of impaired Ptase activity on the transport of MPRs and lysosomal enzymes, we have performed immuno-electron microscopy on a B-cell line derived from an I-cell diseased patient and tissues from Ptase knock out mice. Our findings indicate that loss of Ptase activity results in the accumulation of autophagolysosomes in secretory cells and that the trafficking of MPRs is largely independent of cargo binding.

78/B19

Trafficking of ClC-7 to Lysosomes Controls Lysosomal Acidification in Microglial Cells.

A. Majumdar, D. Cruz, F. Maxfield; Biochemistry, Weill Medical College of Cornell University, New York, NY

Microglia are the phagocytes of the brain, and they play a major role in the clearance of fibrillar forms of Alzheimer's amyloidβ peptide (fAβ). Microglia can efficiently internalize fAβ, but fail to degrade the fAβ because their lysosomes are not properly acidified. Objectives: The main objectives of the present study are to understand the reason for the inefficient acidification of the lysosomes in microglia and to explore the role played by the lysosomal chloride channel ClC-7 in this process. Methods: Experimental techniques used for this study are based on quantitative fluorescence microscopy. Experiments performed include immunofluorescence experiments for studying the localization of ClC-7 in microglia, cellular degradation studies of fluorescently labeled fAβ, and measurement of lysosomal pH in microglia by the use of fluorescently labeled probes. Results: We found that microglia lack ClC-7 in their lysosomes, and that impedes both their lysosomal acidification and their ability to degrade fAβ. ClC-7 is synthesized by microglia, but it is mainly found in aggresomes. Activation of microglia with proinflammatory reagents induces trafficking of CLC-7 to lysosomes and proper lysosomal acidification. The activated microglia can degrade fAβ.The lysosomal recruitment of ClC-7 in microglia is critical for the process of acidification as the extent of lysosomal acidification is correlated with the amount of ClC-7 present in the lysosomes. ClC-7 is often associated with another protein, Ostm1, which plays an important role in its ER exit and lysosomal targeting. Expression of Ostm1 increases in activated microglia, which may account for the increased delivery of ClC-7 to lysosomes. Conclusion: Our findings suggest a novel mechanism of lysosomal pH regulation in activated microglia which is crucial for fAβ degradation.

79/B20

The AP-3 Vesicle Adaptor Binds the HOPS Vacuole Tethering Complex.

C. G. Angers, A. J. Merz; Biochemistry, University of Washington, Seattle, WA

In *Saccharomyces cerevisiae*, the Adaptor Protein complex 3 (AP-3) vesicle adaptor mediates the trafficking of certain cargos from the late Golgi to the vacuolar lysosome. While many of the same machinery are utilized by different vesicle trafficking pathways, it is expected that certain proteins will confer specificity to each step of trafficking. One of the few proteins identified to have a specific role in the AP-3 pathway is Vps41, yet its function remains undefined. We now show that the AP-3 δ subunit, Apl5, binds Vps41 in context of the Homotypic fusion and vacuole Protein Sorting (HOPS) vacuole tethering complex. While Apl5 directly binds to purified Vps41, it preferentially binds native Vps41 in the context of the HOPS complex. This interaction is attenuated by deletion of the HOPS specific subunit, Vps39, but not the Vacuolar Protein Sorting - class C (VPS-C) core subunit, Vps16. Binding of the remaining subunits requires an intact HOPS holocomplex. Moreover, deletion of HOPS subunits or other vacuole tethering/fusion factors results in the subcellular redistribution of AP-3 *in vivo*. We also find that five of the six HOPS subunits, and two closely related class C core Vacuole/Endosomal Tethering (CORVET) subunits, have predicted domain structures that are highly similar to proteins involved in generating membrane curvature, such as vesicle coats or nuclear pore components. Together, these data suggest that the HOPS:AP-3 interaction may couple AP-3 vesicle formation to tethering and fusion at the vacuole.

80/B21

Towards Understanding the Branching of the Vacuolar and Secretory Transport Routes in Plant Cells.

F. Bottanelli, J. Denecke; University of Leeds, Leeds, United Kingdom

The endosomal system of plant cells shows significant unique features that must have emerged after the common eukaryotic ancestor. For instance the small GTPases of the Rab5 family localize to the prevacuolar compartment or late endosome rather than the early endosome. Moreover Rab7 localizes to the vacuolar membrane and not to the late endosome like in mammalian and yeast cells. Plants seem to have evolved differently and the role of Rabs in plant cells can thus not be inferred by homology with the mammalian and yeast proteins. In our study we have analysed plant orthologs of the rab5 (late endosome), rab6 (Golgi apparatus), Rab8 (Golgi apparatus), Rab7 (tonoplast) and rab11 (trans-Golgi network) family in an attempt to dissect the hypothesized branching of the vacuolar sorting route from the secretory one in plants. Dominant negative mutants with altered nucleotide binding and/or hydrolysis were created and expressed in plant cells together with different vacuolar and secretory cargo models. Rab5 and Rab7 caused induced secretion of soluble vacuolar cargo leaving the secretory route unaltered but only Rab7 caused a transmembrane tonoplast protein to be blocked en route to the vacuole. Blocking of secretion was not feasible with any of the individual Rab mutants, but could only be achieved by combinations of at least two different Rab mutants. The results suggest that transport to the plasma membrane can occur via different routes but merely suggests a single route to the vacuoles.

81/B22

Essential Role of Snapin in Coordinating Late Endocytic Trafficking and Autophagy-Lysosomal Function.

Q. Cai, L. Lu, J. Tian, Z. Sheng; NINDS/NIH, Bethesda, MD

Lysosomes are dynamic organelles that receive and degrade macromolecules and organelles from secretory, endocytic, and autophagic pathways during cellular homeostasis in health and disease. Although some features of late endocytic trafficking have been described, regulation of this process remains to be elucidated. Using a genetic mouse model, we discover that Snapin acts as an essential component of the late endocytic trafficking machinery and plays a critical role in tightly coordinating late endocytic trafficking and autophagy-lysosomal funciton. First, the deletion of the snapin gene in mice results in a robust accumulation of autolysosome in snapin-deficient hippocampal slices, cultured cortical neurons, and mouse embryonic fibroblasts (MEFs) under both non-starved and starved conditions. This phenotype can be effectively rescued by transient expression of Snapin in the mutant cells. Second, deletion of snapin results in an abnormal peri-nuclear accumulation of late endosomes in cultured mouse cortical neurons and MEFs. Snapin gain-of-function could effectively induce conversion of late endosomes into mature lysosomes. Third, pulse-chase studies in live cells combined with light and electron microscopy observations demonstrate that snapin deficiency significantly results in impaired degradation of internalized EGF and BSA-gold, retention of internalized Dextran in late endosomes rather than delivery to lysosomes, and reduced maturation of lysosomal enzyme cathepsin D. Finally, using time-lapse imaging in live cells, we further demonstrated that elevated expression of Snapin enhances dynamic late endosome-lysosome trafficking and facilitates the formation of tubular structures that emanate from lysosomes and late endosomes. Thus, our studies indicate that Snapin facilitates the late endocytic pathway, thereby highlighting a mechanistic insight into regulation of autophagy-lysosomal function. Such a mechanism is critical to maintain maturation and proper function of lysosomal system, and thus contributes to the complex regulation of multiple degradation pathways including autophagy. (Supported by the Intramural Program of NINDS, NIH).

82/B23

Genetic Analysis of Biogenesis of Lysosome-related Organelles Complex-1 (BLOC-1) Subunit 1 (Blos1) in Drosophila melanogaster.

V. T. Cheli[1], R. Godoy[2], I. A. Rodriguez-Fernandez[1], V. K. Lloyd[2], D. E. Krantz[3], E. Dell'Angelica[1]; [1]Department of Human Genetics, University of California Los Angeles, Los Angeles, CA, [2]Department of Biology, Mount Allison University, Sackville, NB, Canada, [3]Psychiatry and Biobehavioral Sciences, University of California Los Angeles, Los Angeles, CA

Hermansky-Pudlak syndrome (HPS) is a group of human genetic disorders characterized by abnormal biogenesis of lysosome-related organelles (LROs), which results in manifestations such as hypopigmentation in eyes and skin and prolonged bleeding. Consistent with a conservation of molecular mechanisms involved in the biogenesis of LROs in mammals and insects, several of the genes associated with HPS have orthologues in the fruit fly, *Drosophila melanogaster*, belonging to the "pigment granule" group of genes required for the formation of an insect LRO: the eye pigment granule. These include the *garnet, ruby, carmine* and *orange* genes encoding subunits of adaptor protein-3 (AP-3) and the *pink* gene encoding a subunit of biogenesis of lysosome-related organelles complex (BLOC)-2. Another protein complex associated with HPS in mammals is BLOC-1, however no mutation in a fly BLOC-1 subunit has been described to date. In this work, we have generated two null mutant alleles of the *CG30077* gene encoding the only fly counterpart of BLOC-1 subunit 1 (BLOS1). These flies showed a significant (~70%) reduction in the levels of both types of eye pigments (red and brown), a phenotype that is characteristic of members of the pigment granule group. Epistatic analyses demonstrated phenotypic enhancement with mutant alleles in *garnet, ruby* and *carmine* as well as in *lightoid*, another classical pigment granule group gene. Interestingly, *CG30077* mutant flies displayed increased male-

to-male courtship behavior, which like that previously observed for *garnet* mutant flies was strictly dependent on the function of the *white* gene and might reflect a role for *CG30077* (and, by extension, fly BLOC-1) in the intracellular trafficking of the *white* gene product. These results suggest that BLOC-1 belongs to the conserved molecular machinery that participates in the biogenesis of LROs in both insects and mammals.

83/B24

Novel Pathways for Lysosomal Delivery - Analyses of the Intracellular Trafficking Itineraries of the Recycling Receptor, FcRn, in Live Cells.

Z. Gan[1,2], S. Ram[1], C. Vaccaro[1], R. Ober[1,3], E. Ward[1]; [1]Immunology, UT Southwestern Medical Center, Dallas, TX, [2]Biomedical Engineering Program, UT Southwestern Medical Center, Dallas, TX, [3]Department of Electrical Engineering, University of Texas at Dallas, Richardson, TX

Lysosomes play a central role in the degradation of proteins and other macromolecules. The mechanisms by which receptors are transferred to lysosomes for constitutive degradation are poorly understood. We have analyzed the processes that lead to the lysosomal delivery of the neonatal Fc receptor, FcRn. FcRn is a specific receptor for IgG and is ubiquitously expressed in many cell types. Structurally and functionally, FcRn is distinct from the other classical Fc receptors and is responsible for regulating and transporting IgG in the body. Our studies provide support for a novel pathway for receptor delivery. Specifically, unlike other receptors that enter intralumenal vesicles in late endosomes, FcRn is transferred from the limiting membrane of such endosomes to lysosomes, and is rapidly internalized into the lysosomal lumen. By contrast, LAMP-1 persists on the limiting membrane. Receptor transfer is mediated by tubular extensions from late endosomes to lysosomes or by interactions of the two participating organelles in kiss-and-linger like processes, whereas full fusion is rarely observed. The persistence of FcRn on the late endosomal limiting membrane, together with selective transfer to lysosomes, allows this receptor to undergo recycling or degradation. Consequently, late endosomes have functional plasticity, consistent with the presence of the Rab5 GTPase in discrete domains on these compartments.

84/B25

Role of the Vps4 ATPase in the MVB Sorting Pathway.

A. M. Shestakova, M. Babst; University of Utah, Salt Lake City, UT

Cell surface receptors and other transmembrane proteins are regulated, in part, by the vacuolar/lysosomal degradation pathway. Monoubiquitinated transmembrane proteins are trafficked to the vacuole/lysosome via the Multivesicular Body (MVB) sorting pathway. Formation of the MVBs is regulated by three Endosomal Sorting Complex Required for Transport (ESCRT) protein complexes and the Vps4 ATPase. During MVB formation the ESCRT machinery assembles transiently on the MVB membrane to sort cargo into inward budding vesicles, and is subsequently disassembled and recycled by the activity of Vps4, directly recruited by ESCRT-III. We use the yeast Saccharomyces cerevisiae to study the interactions within the Vps4 homooligomer and between the Vps4 homooligomers and ESCRT-III. Our studies indicate that the localization of Vps4 to ESCRT-III is mediated by interactions 1) between Vps4 subunits within a homooligomer; 2) between Vps4 and the regulatory protein Vta1; and 3) between Vps4 and the ESCRT-III subunits Vps2/Vps24. Previous in vitro studies by others suggest that Vps4 oligomerizes into a double-ring structure in a tail-to-tail manner with one of the rings contacting ESCRTIII. In agreement, we report that interfering with the C-termini of Vps4 monomers destabilizes double-ring assembly in vivo, and that Vta1 is not required for the assembly of the Vps4 double-ring homooligomer in vivo. Moreover, we demonstrate that in vitro the single-ring Vps4 structure is capable of ATP hydrolysis, and present data suggesting that ESCRT-III disassembly requires ATP hydrolysis only by the ESCRT-III-associated Vps4 ring indicating different functions for the two rings. Based on our findings, we propose that Vps4 homooligomerizes on the MVB membrane by assembling a substrate-interacting ring first, followed by the addition of the second ring.

85/B26

A Genomic Screen to Identify Genes Involved at the Endosome and Vacuole Interface in Baker's Yeast.

R. Menjivar, M. Loayza, S. Chhun, E. Gharakhanian; Biological Sciences, CSULB, Long Beach, CA

Our laboratory is interested in gene functions involved in lysosomal trafficking, morphology, and function. The objective of this project is to identify genes involved in vacuolar events at the late endosome to lysosome/vacuole interface by a genome-wide screen of a MAT-α deletion strain of non-essential genes in Saccharomyces cerevisiae. The deletion strain library was subjected to an immunodetection screen developed in our laboratory. We have screened the entire library using our primary immunodetection method. From the primary screen, 200 mutants were identified which displayed defects in processing of carboxypeptidase Y. The uncovered mutants are being subjected to a secondary immunodetection screen to identify mutants that internally accumulate pro-CPY. To date, we have identified eight deletion mutants that internally accumulate pro-CPY, known as env (endosome to vacuole) mutants. The uncovered mutants include one major group of uncharacterized genes and genes involved in other cellular processes such as cell cycle control and osmoregulation. Further characterization of the env mutants are currently in progress. Moreover, we have identified five additional mutants that secrete p2CPY that, to date, have not been reported as vacuolar protein sorting (vps) mutants. The genomic approach allows the identification of a collection of ENV genes which will provide a bigger picture of the gene functions involved at the late endosome and lysosome interface. This in turn will provide a better understanding of the final steps in yeast CPY pathway and the mammalian M6P pathway and may ultimately reveal how defects in the M6P pathway leads to lysosomal storage and neurodegenerative diseases.

86/B27

Reconstitution of Multivesicular Body Formation.

J. Tran, R. Schekman; Dept. of Molecular & Cell Biology, University of California, Berkeley, Berkeley, CA

The multivesicular body (MVB) pathway involves transport of transmembrane proteins to the vacuole lumen in vesicles internalized by an endosome. Sorting and internalization require cytosolic ESCRT complexes that recognize ubiquitinated cargo on endosomal membranes. The ESCRTs were identified in genetic screens as being essential for the packaging of cargo and for the formation of luminal vesicles in

endosomes. Vps27p is the first ESCRT protein to recognize ubiquitinated cargo. At the end of this process, the ATPase Vps4p removes the ESCRTs from the endosome surface for subsequent rounds of invagination. However, the mechanism of cargo segregation and internal vesicle formation has yet to be elucidated. We have established a biochemical assay using membranes and cytosol extracted from *Saccharomyces cerevisiae* to reconstitute this reaction *in vitro* to identify the minimal components necessary for vesicle formation. Using a *vps27-ts* strain grown at a nonpermissive temperature, we have generated endosomes accumulated with a transmembrane cargo, the protease carboxypeptidase S (CPS), trapped prior to internalization. The cytosolic tail of CPS was fused to tandem biotin acceptor peptides that are specifically biotinylated *in vitro* by *E. coli* biotin ligase. Biotin conjugated to CPS, marking CPS at the endosome surface, is completely removed by trypsin, whereas an HA tag fused to the luminal portion of CPS was not cleaved by trypsin, demonstrating that CPS was trapped on an intact endosome. Cytosol extracted from a wild-type (WT) strain and incubated with *vps27-ts* membranes was shown to elicit protection of CPS-biotinylated cargo from the action of trypsin, indicative of CPS sorting into internal vesicles. Mutant cytosol from *vps27Δ*, *vps4Δ*, and *vps27Δ vps4Δ* strains elicited reduced CPS protease protection relative to WT cytosol, demonstrating the importance of Vps27p and Vps4p in the pathway. CPS protection with WT cytosol was suppressed at 4C and inhibited by addition of apyrase, indicating a physiological and ATP-dependent reaction, but not by addition of a nonhydrolyzable GTP analog, GTPγs. Addition of recombinant Vps27p without cytosol rescued *vps27* mutant membranes and not *vps4* mutant membranes.

86A/B28

Investigation of Endosome/MVB Pathway-mediated Regulation of Alpha-Synuclein Localization, Accumulation, and Toxicity in a Yeast Model Reveals Unexpected Complexity.

A. Ayala, M. White, S. DebBurman; Lake Forest College, Lake Forest, IL

Misfolding, accumulation, and aggregation of the alpha-synuclein protein is linked to the pathogenesis of Parkinson disease (PD). Enhancing alpha-synuclein degradation may help prevent its accumulation and aggregation, and protect cells against its toxicity. Pharmacological studies suggest that the proteasome and lysosome both serve as degradation sites for alpha-synuclein, however, genetic evidence is still lacking for the latter. Thus, our goal was to seek genetic evidence for alpha-synuclein degradation via the lysosome. We used an alpha-synuclein budding yeast model, and evaluated alpha-synuclein localization, accumulation, and toxicity in gene knockout strains compromised for the endosome/multivesicular body (MVB) pathway, a major route used by yeast to target proteins for degradation in the vacuole. Specifically, we utilized strains that eliminated specific proteins that are components of ESCRT complexes (I, II, and III) that mediate the MVB pathway. From the strains analyzed thus far, none enhanced alpha-synuclein-dependent toxicity. However, several altered alpha-synuclein localization within cells to various degrees. The absence of vps28 (which forms ESCRT-I) was dramatic; alpha-synuclein relocalized from the plasma membrane to intracellular locales, including the formation of punctate foci near the vacuole. The absence of vps24 (ESCRT-III) was more subtle; alpha-synuclein localization at the plasma membrane was less intense, with an increase in overall cytoplasmic localization. In contrast, the absence of other proteins (ESCRT-I members mvb12 and vps23, ESCRT-II member vps25) negligibly affected alpha-synuclein localization. While our data indicates that the MVB pathway may be a route for alpha-synuclein degradation, the extent of involvement of proteins within the ESCRT complexes with alpha-synuclein appears unexpectedly complex. To fully understand the MVB-mediated alpha-synuclein degradation, we are currently analyzing knockout strains for the rest of the proteins that comprise the Endosome/MVB pathway. (Supported by NSF-MRI, NSF-CCLI & NIH R15)

Extracellular Matrix and Cell Signaling (87-112)

87/B29

Role of ICAP-1 and Rho A Kinase IN B1-Integrin Mediated Regulation of Homologous Recombination In the Epithelial Breast Cells.

N. Heidari, A. Rizki; Radiation Oncology Department, Virginia Commonwealth University, Richmond, VA

Normal breast tissue is composed of a double-layer of epithelial tissue surrounded by a basement membrane which is a specialized extracellular matrix (ECM) rich in laminin. ECM interaction within the tissue context affects cell functional integrity, including regulation of cell cycle, apoptosis, differentiation as well as the functional organization of the nucleus and the stability of the genome. Our data show that ECM signaling through β1-Integrin regulates the HR of an endonuclease-induced double-strand break (DSB) within a direct repeat substrate as well as the foci formation kinetics of the DSBR proteins H2AX, MRE11, and RAD51.To delineate the cytoplasmic signaling events that relay ECM signals to nuclear DNA repair processes we are determining the role of ICAP-1 (Integrin cytoplasmic domain-associated protein-1) which is the first protein found to inhibit β1-integrin function by preventing its cytoplasmic tail from interacting with downstream effectors. For this specific aim we studied ICAP-1 expression under the effect of ECM using QPCR and western blotting in cells cultured on Matrigel.Data show that ICAP-1 expression change in 3D culture and under the effect of ECM. To determine the effect of ICAP-1 expression on HR we knocked out ICAP-1 in breast epithelial cells and determined its effect on DSBR using endonuclease-induced DSB within the direct repeat substrate as well as foci formation kinetics of H2AX, MRE11, and RAD51 in irradiated cells. All results confirmed by ICAP-1 overexpression in epithelial breast cells. We also determined the cytoplasmic localization of ICAP-1 and we are investigating the shutting of this protein in nucleus under the effect of ECM. To delineate the signaling pathway of β1-Integrin mediated homologous recombination we also investigate the role of RhoA kinase using RhoA kinase inhibitors. Since previous data showed that ICAP-1 interact with ROCK-I which is one of the effectors of RhoA, we are looking for the role of RhoA kinase in ICAP-1 mediated signaling pathways. Determining which ECM signaling components regulate DSBR in normal cells can provide targets and markers relevant to carcinogenesis and radiation response.

88/B30

The Interaction of β-catenin with Extra-cellular Matrix Components in Dermal Fibroblasts during Wound Healing.

K. A. Bielefeld[1,2], S. Amini Nik[1], B. Alman[1,2]; [1]Developmental and Stem Cell Biology, Hospital for Sick Children, Toronto, ON, Canada, [2]Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada

During cutaneous wound healing, dermal fibroblasts synthesize new extracellular matrix (ECM) to repair the skin. β-catenin binds and stimulates tcf transcription factors, and it is activated in wound fibroblasts. However, the factors that regulate β-catenin during healing, and its transcriptional targets during this process, are not well understood. One possibility is the involvement of the ECM as both a target and a regulator of β-catenin activity. We investigated the existence of a reciprocal regulatory loop between β-catenin and ECM during healing. The expression pattern of several ECM components, which include β-catenin transcriptional targets, was verified using real time PCR analysis in mouse full thickness wounds. To investigate the effect of ECM on β-catenin, primary dermal fibroblasts from tcf-reporter mice were cultured on collagen I, IV, fibronectin, and matrigel, and analyzed for protein expression and tcf-dependent transcriptional activity by immunoblotting and β-galactosidase assay, respectively. We found an increase in activated β-catenin protein and in tcf-dependent transcription in fibroblasts grown on ECM compared to plastic. Inhibition of glycogen synthase kinase 3β, which mediates the destruction of β-catenin, was demonstrated by immunoblot analysis in fibroblasts cultured on ECM. Treatment of dermal fibroblasts with the ECM-integrin binding motif, RGDS, caused an elevation in active β-catenin protein. To identify ECM-associated targets of β-catenin during wound healing, we used expression array analysis to compare gene expression in mice with conditionally regulated β-catenin versus normal levels: numerous ECM and adhesion molecules were differentially expressed in response to conditional stabilization of wound β -catenin. Thus, ECM upregulates β-catenin protein and transcriptional activity and β-catenin modulates the expression of ECM components, suggesting that the ECM acts in a feedback loop during wound repair to regulate β-catenin. Elucidating the mechanism of this regulation will enhance our understanding of signaling between the intra- and extra-cellular environments in the response to injury.

89/B31

Difference in Neutrophil Motility induced by a Novel Superstructure of Collagen.

S. Kunii, K. Morimoto; Biotechnological Science, Kinki University, Kinokawa, Japan

[Objective] Recently, it was shown that immune cells such as neutrophil interact with type I collagen using leukocyte-associated Ig-like receptor-1 (LAIR-1). Neutrophil transmigrates through blood vessel wall and migrates into an extracellular matrix toward an inflammatory site, as a vital first immune response. In the motility and the functions of the neutrophil, Rho family proteins play a crucial role. However, how the neutrophil motility is regulated by the collagen in the extracellular matrix remains unclear. Until now, we have shown that type I collagen hydrolyzed by actinidain (AP-collagen) changes conformation and the motility of neutrophil. In this study, we investigated the expression of actins, Cdc42, RhoA, and Rac1 of neutrophils induced by the AP-collagen. [Methods] We prepared pepsin-hydrolyzed type I collagen (atelocollagen) and AP-collagen from chicken skin. Neutrophil was collected from a mouse by casein injection into a peritoneal cavity, and subsequently was cultured on a plate coated with the atelocollagen or the AP-collagen. To investigate neutrophil motility cultured on the collagen matrix, we observed F-actin labeled with MFP-555-Phalloidin by using fluorescence microscopy. To find further evidence of the actin activation, we visualized Rho family proteins in the neutrophils by staining with specific antibodies. [Results] The fluorescence observations revealed that the expressions of Rho family proteins of neutrophils on the AP-collagen or the atelocollagen matrix are clearly different. Therefore, neutrophil would recognize differences in conformation between the AP-collagen and the atelocollagen as the scaffold. [Conclusions] In conclusion, we found that the AP-collagen alters intracellular signaling pathways of neutrophil and induces its specific function.

90/B32

Matrix Density-induced Stiffness Influences Breast Cell Phenotype, Signaling, and Gene Expression through a FAK-ERK Linkage.

P. Provenzano, D. Inman, K. Eliceiri, P. Keely; Laboratory of Molecular Biology, University of Wisconsin, Madison, WI

Dense breast tissue is linked to a greater than 4-fold increased risk of breast carcinoma. Importantly, areas of increased breast density are not only associated with increased cellularity, but also significantly increased collagen deposition. Yet, although a link between increased breast tissue density and breast cancer has been established, the molecular mechanisms driving the effects of the extracellular matrix on breast carcinoma development are not known. Our hypothesis is that increased breast density is associated with a rigid and re-organized extracellular matrix, resulting in high local mechanical loads for breast epithelial cells, which ultimately leads to enhanced 3D matrix adhesion formation and signaling. This hypothesis is supported by data showing that FAK and ERK phosphorylation increases following tensile loading on 2D substrates or within 3D collagen matrices. Moreover, increasing the density and/or stiffness of a 3D collagen matrix alters cell morphology, increases Rho activity and stress fiber formation, and promotes FAK and ERK phosphorylation at matrix adhesions. Combined, these altered signaling events contribute to a shift in the transcriptome resulting in a gene expression pattern that is indicative of more proliferative and invasive phenotype. In particular, increased extracellular matrix stiffness promotes proliferation and enrichment of a proliferation gene signature that can be reverted with inhibition of ERK. In fact, ablating ERK phosphorylation reverts ~70% of the genes regulated by matrix stiffness, raising the possibility that ERK acts as a primary node, or bottleneck, in the signaling network activated by mechano-signaling through epithelial cells. Interestingly, blocking ERK phosphorylation with an inhibitor of MEK also reverts the invasive phenotype associated with dense/stiff matrices in a similar manner as inhibition of the Rho/ROCK pathway, and ERK phosphorylation is FAK/Src dependent. Together, these data imply that epithelial cell behavior and proliferation in response to matrix stiffness is regulated via integrin/focal-adhesion pathways, and that these pathways converge on ERK as the primary regulator of the cellular response to mechanical signals.

91/B33

An Integrated Response Mechanism That Encompasses Cell and Extracellular Matrix Mechanics Regulates Integrin Binding Cooperativity, Clustering, and Adhesion Function.

M. J. Paszek[2,1], D. Boettiger[3], D. A. Hammer[2], V. Weaver[1,2]; [1]Surgery, University of California - San Francisco, San Francisco, CA, [2]Bioengineering, University of Pennsylvania, Philadelphia, PA, [3]Microbiology, University of Pennsylvania, Philadelphia, PA

Integrins are part of a cellular-environment sensory machine that responds to chemical and physical extracellular matrix (ECM) cues by clustering into adhesion plaques with modified signaling functions. However, the "force sensors" and "molecular cross-linkers" that sense ECM cues to modulate integrin clustering have yet to be identified. We developed an advanced spatial-temporal simulation that integrates the

micro-mechanics of composite elastic materials at the cell-ECM interface with a simple chemical model of integrin activation and ligand interaction. Using this model we show that integrins possess innate properties that permit them to cluster in response to biochemical and biophysical cues received from the ECM. We predict that due to mechanical coupling, integrin-ligand interactions are highly cooperative with Hill coefficients that can approach or exceed those reported for ultra-sensitive signaling cascades. Through this cooperativity, integrin clustering appears to be driven solely by ligand binding interactions, yet remains highly responsive to ECM rigidity and ligand spacing. Use of live cells or "cell-free" plasma membranes interacting with deformable ECM substrates demonstrated that ligand binding is sufficient to trigger integrin clustering provided the ECM is sufficiently rigid. In addition, although we maintain that cytoskeletal forces are likely dispensable for integrin clustering and for interrogating ECM properties, our model nonetheless predicts that small contractile forces invariably augment integrin clustering by enhancing cell and ECM material deformations. Thus we could show that sub-pN forces applied to bound integrin receptors with a spinning disk device could stimulate integrin clustering before discernable recruitment of additional adhesion complex components. We also showed that expression of an auto clustering V737N integrin mutant could recapitulate integrin clustering on a compliant substrate. Provocatively, the model predicts that alterations in cellular and glycocalyx mechanics, as have been documented in metastatic tumor cells, would hypersensitize the integrin adhesion system to changes in ECM dynamics; a possibility we are now testing. (DOD W81XWH-05-1-330 to VMW & DAH)

92/B34

Endothelial Cells Orient to Surface Topography through Localized Changes in Focal Adhesion Kinase Signaling.

J. Gasiorowski[1], P. F. Nealey[2], C. J. Murphy[1]; [1]Surgical Sciences, University of Wisconsin, Madison, WI, [2]Chemical and Biological Engineering, University of Wisconsin, Madison, WI

Cells in vivo are never exposed to the flat surfaces common to most tissue culture plasticware. Instead, cells within native tissues rest on topographically rich, 3-dimensional surfaces. Our group has fabricated topographically patterned, anisotropically ordered substrates possessing, nano- through micro-scale feature dimensions. These have served as tools to elucidate the mechanisms by which physical surface cues are translated into biochemical signals that direct changes in cell behavior. Human Umbilical Vein Endothelial Cells (HUVECs) align and orientate parallel to the grooves and ridges of our substrates within 2 hours of plating. However, there were clear differences in focal adhesion kinase (FAK) localization after 30 minutes when comparing HUVECs grown on either topographically patterned or flat control substrates with identical surface chemistry. At 30 minutes post plating, immunofluorescence images showed that activated FAK, phosphorylated at tyrosine 397, localized into elongated linear structures at the edges of the substrate ridges. In contrast, the same activated FAK was found in small speckles at the cell periphery when HUVECs were plated onto flat surfaces. Other focal adhesion proteins such as Src and p130Cas were also phorphorylated at tyrosine residues and co-localized with activated FAK in the linear structures. Immunofluorescence images of HUVECs plated onto the flat control substrates showed Src and p130Cas were phosphorylated with a random, speckled localization at the cell periphery. Additionally, phalloidin-stained actin stress fibers orientated parallel to the ridges and grooves 60-120 minutes after the HUVECs were plated. The dramatic positional changes in this group of activated focal adhesion proteins suggest they mediate the signaling cascades that re-orient the actin cytoskeleton parallel to the ridges and grooves of the substrate. These data contribute to our understanding of signaling pathways that mediate a cellular response to biophysical cues and can help energize new development strategies in cell, stem cell and tissue engineering.

93/B35

Rac1 Is a Substrate for Non-Receptor Tyrosine Kinases FAK and Src.

F. Chang[1], L. Romer[1,2,3]; [1]Anesthesiology and Critical Care Medicine, Johns Hopkins University School of Medicine, Baltimore, MD, [2]Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD, [3]Biomedical Engineering, Pediatrics, and the Center for Cell Dynamics, Johns Hopkins University School of Medicine, Baltimore, MD

Integrins regulate cell behavior through recruitment and activation of signaling proteins at sites of cell-matrix adhesion. Two of these signaling elements, Src and FAK, are non-receptor cytoplasmic tyrosine kinases that may act cooperatively during the regulation of cell spreading and migration. Previously, we showed that FAK and Src can tyrosine phosphorylate beta-PIX, a Rac/Cdc42-specific GEF, to induce Rac1 activation and promote cell spreading. Our new studies have demonstrated that FAK and Src directly phosphorylate Rac1 at Y64. A Y-F point mutation at this position abolished tyrosine phosphorylation of Rac1 by FAK and Src, despite the presence of seven other tyrosine residues in Rac1. This result indicates that Y64 is the major phosphorylation target for FAK and Src. Furthermore, both Y-F and Y-D point mutations at position 64 alter cell spreading. MEF expressing Rac1-Y64F mutant spread better than the wild type controls, whereas cells expressing Y64D mutant show only limited spreading. These changes may be due to the abrogation of Rac1 tyrosine phosphorylation by the 64F mutation, and by mimicking of a constitutively phosphorylated state by the 64D mutation. Additional Rac1 mutants have been constructed to test the interaction between FAK- or Src-mediated tyrosine phosphorylation and other regulatory inputs for Rac1 function.

94/B36

Quantitative Model of Leading Edge Dynamics Based on Rac/Pak Signaling.

M. Krajcovic, M. Cirit, J. Haugh; Chemical & Biomolecular Engineering, North Carolina State University, Raleigh, NC

The mechanisms governing the formation and disassembly of integrin-extracellular matrix (ECM) adhesion complexes at the protruding leading edge are central to the understanding of cell migration. Active Rac promotes protrusion, accompanied by the formation of nascent focal adhesions. Nascent adhesions either turn over or mature into stable focal adhesions; these two types of adhesion complexes are readily distinguished by total internal reflection fluorescence microscopy of GFP-paxillin-expressing cells. Activation of p21-activated kinase (Pak) is mediated by nascent adhesions through a positive feedback loop involving phosphorylation of paxillin Ser273 by Pak and recruitment of Git1-Pix-Pak complexes, which in turn mediate activation of Rac and Pak. Pak activity promotes turnover of nascent adhesions locally, whereas maturation of nascent adhesions is promoted in a more global fashion by actomyosin-mediated contractility. We have developed a model that describes in mathematical terms the feedback loops driving, on the one hand, Rac-mediated membrane protrusion and rapid turnover of the nascent adhesions, and on the other, myosin-dependent maturation of adhesions that promote additional contraction. We find

that the system is naturally bistable; that is, it produces two stable steady states for the same ECM density. The biological implication of this result is that one will tend to find highly protrusive regions with mostly nascent adhesions, as well as spatially fixed regions with stable adhesions, and this bistable nature should be most prevalent at intermediate ECM density. Spatially explicit, stochastic simulations of the system reveal dynamic plumes of signaling and protrusion activity, reminiscent of experimental observations.

95/B37

Connective Tissue Growth Factor (CTGF/CCN2) as a Regulator of β1 Integrin-mediated Cell Adhesion and Survival during Lactogenic Differentiation of Mouse Mammary Epithelial Cells.

B. Morrison[1], C. Jose[1], N. Kenney[2], M. Cutler[1]; [1]Dept. of Pathology, Uniformed Services University, Bethesda, MD, [2]Dept. of Biological Sciences, Hampton University, Hampton, VA

Mammary epithelial cell differentiation is dependent on the response to basement membrane and stromal protein-induced signals. Our previous work determined that connective tissue growth factor (CTGF/CCN2), a known stromal mediator, was highly up-regulated during lactogenic differentiation of mouse mammary epithelial cells. CTGF/CCN2 is transcriptionally regulated by dexamethasone and is required for lactogenic differentiation of the HC11 mouse mammary epithelial cell line. CTGF/CCN2 expression, regulated by a Tet-off system in the HC11 cell background, both enhanced cell growth and survival and prevented apoptosis of mammary epithelial cells. HC11 cells expressing CTGF/CCN2 exhibited increased levels of β1 integrin and downstream mediators including phospho-FAK, phospho-Akt, and cyclin D1. CTGF/CCN2 increased the formation of focal adhesions, as viewed by immunofluorescence, and increased the levels of focal adhesion-related adaptor, structural, and signaling proteins including p130cas, paxillin, Src, vinculin, parvin and ILK. CTGF/CCN2 expression enhanced multiple markers of lactogenic differentiation including β-casein transcription, stabilization of Stat5 phosphorylation and mammosphere formation. Infection of primary mouse mammary epithelial cells with replication defective adenovirus encoding CTGF/CCN2 induced similar responses. Elevated CTGF/CCN2 expression decreased the requirement for extracellular matrix components including collagen I, fibronectin, and laminin for initiation of β-casein transcription. Because β1 integrin signaling is required for lactogenic differentiation and the transcription of β-casein, the contribution of CTGF/CCN2 to the formation and stability of the β-casein promoter transcription complex has been examined by chromatin immunoprecipitation. The effect of CTGF/CCN2 on both Stat5 and the SWI-family protein, Brg1, binding and function at the β-casein promoter were examined. These results demonstrate that the mechanism by which CTGF/CCN2 contributes to lactogenic differentiation is via activation of β1 integrin-mediated adhesion complexes and integrin-dependent signaling pathways enhancing both cell survival and the transcriptional changes required for lactogenesis.

96/B38

The Matricellular Protein CCN1 Enhances Fas-mediated Apoptosis In Vitro and In Vivo.

V. Juric, C. Chen, L. Lau; Departemnt of Biochemistry and Molecular Genetics, University of Illinois at Chicago, Chicago, IL

The extracellular matrix (ECM) is known to profoundly regulate cell survival. To uncover how the ECM microenvironment modulates apoptosis triggered by cell surface 'death receptors,' we tested the apoptotic response to Fas ligand (FasL) in cells adhered to various ECM proteins, including the matricellular protein CCN1. CCN1 is a secreted integrin-binding ligand whose expression is highly regulated during tissue remodeling and inflammation, and is essential for cardiovascular development. Exposure of fibroblasts to purified CCN1 protein either as an adhesion substrate or a soluble factor significantly enhances FasL-induced apoptosis, whereas other ECM proteins tested have little effect. CCN1 engagement to its adhesion receptors, integrin α6β1 and heparan sulfate proteoglycans (HSPGs), is required to synergize with FasL. Neither the expression nor cell surface localization of the Fas receptor, which initiates the apoptotic pathway, is altered by CCN1. Mechanistically, CCN1 induces the accumulation of intracellular reactive oxygen species (ROS) via pathways dependent on neutral sphingomyelinase-1 and 5-lipoxygenase, as inhibition or knockdown of these enzymes abrogated ROS as well as the apoptotic synergism of Fas/CCN1. Fas-mediated p38 MAPK activation, which is critical for Fas/CCN1-induced apoptosis, is amplified by CCN1 in a ROS-dependent manner. In addition, CCN1 augments activation of caspases 8, 10, 9, and 3 by Fas, each of which is required for cell death. To determine whether Fas/CCN1 synergism occurs in vivo, we used knockin mice in which the wild-type Ccn1 allele was replaced by an α6β1/HSPG-binding-defective mutant. Apoptosis in liver was induced by i.v. delivery of the agonistic antibody against Fas, Jo2. The mutant mice are significantly resistant to Jo2-induced apoptosis as compared to wild-type mice, and exhibit substantially reduced oxidative DNA damage. Together, these results show that apoptosis via death receptors can be regulated by integrin-mediated signaling, and that the matricellular protein CCN1 may act as a physiologic regulator of Fas-mediated cell death.

97/B39

Regulation of Epithelial-Mesenchymal Transition and Fibrogenesis by Mechanical Signals from the Microenvironment.

E. W. Gomez[1], D. Radisky[3], C. Nelson[1,2]; [1]Chemical Engineering, Princeton University, Princeton, NJ, [2]Molecular Biology, Princeton University, Princeton, NJ, [3]Cancer Biology, Mayo Clinic Cancer Center, Jacksonville, FL

Fibrosis, a pathological condition that arises from aberrant wound healing processes, can affect a variety of tissues including lung, liver, and breast and is characterized by stiffening of the extracellular matrix and disrupted tissue architecture. Recent studies have shown that myofibroblasts, key players in both normal wound healing and fibrosis, can be derived from epithelial cells through an epithelial-mesenchymal transition (EMT) induced by matrix metalloproteinase-3 (MMP-3). MMP-3 induces EMT, fibrosis, and tumor formation in the mammary gland in vivo and in cultured mammary epithelial cells. We show here that cell shape and matrix compliance are critical determinants of mammary epithelial cell fate in response to MMP-3. We show that morphogenic alterations are a requirement for MMP-3-induced EMT, as limiting the ability of the cells to spread by culturing cells on micropatterned substrata or by increasing cell density blocks MMP-3-induced EMT. We also find that altering the extracellular matrix compliance or composition affects response to MMP-3. Our findings show how normal tissue compliance is protective against MMP3-induced EMT and provide insight towards identification of therapeutic targets for blocking MMP-mediated fibrosis and malignancy in vivo.

98/B40

JNK2 Sulfenic Acid Formation in the Redox Regulation of α5β1 Integrin Signaling.

E. A. Erickson[1], C. M. Furdui[1], R. Yammani[1], A. W. Tsang[1], J. S. Fetrow[2], L. B. Poole[3], R. F. Loeser[1]; [1]Dept. of Molecular Medicine, Wake Forest University School of Medicine, Winston Salem, NC, [2]Dept. of Physics, Wake Forest University, Winston Salem, NC, [3]Dept. of Biochemistry, Wake Forest University School of Medicine, Winston Salem, NC

Reactive oxygen species (ROS) can act as secondary messengers in signaling cascades through the direct oxidation of reactive cysteine (cys) residues, which results in sulfenic acid formation and modified protein activity. We have previously shown fibronectin fragment (FN-f) stimulation of the α5β1 integrin results in an increase in ROS that is essential for the pathway's signal transduction and downstream matrix metalloproteinase (MMP) expression in chondrocytes. The objective of the current study was to use an activity based proteomics approach to determine key signaling intermediates regulated by ROS in the α5β1 integrin signaling pathway. To identify proteins containing oxidized cys residues after FN-f stimulation, cell lysates were incubated with DCP-biotin, a reagent that reacts specifically and irreversibly with sulfenic acids. DCP-biotin labeled proteins were isolated with streptavidin beads and analyzed by SDS-PAGE. Subsequent immunoblotting determined that one of the labeled proteins was c-jun N-terminal kinase 2 (JNK2). To identify the oxidized cys residue(s) within JNK2, purified JNK2 incubated with hydrogen peroxide and DCP-biotin was analyzed by mass spectrometry. Cys^{222} was the only JNK2 cys residue found to form a sulfenic acid. Thus, JNK2 Cys^{222} was changed to a serine to generate a redox-insensitive JNK2 mutant for further examination. To determine whether sulfenic acid formation is required for JNK2 activity, wildtype and mutant JNK2 were transfected, immunoprecipitated from FN-f stimulated chondrocytes, and tested in a JNK kinase assay. The kinase activity of the JNK2 Cys^{222} mutant was substantially lower than that of the wildtype JNK2. In contrast, both the wildtype and Cys^{222} mutant JNK2 showed equal phosphorylation upon stimulation. In summary, these results have demonstrated for the first time that JNK2 acts as a redox-sensitive signaling mediator in the α5β1 integrin pathway and thereby contributes to the ROS regulation of chondrocyte MMP production. Further investigation of JNK2 and other redox-sensitive signaling intermediates that regulate MMP production could aid in the identification of potential therapeutic targets that can slow or stop cartilage degradation in arthritis patients.

99/B41

Characterizing the Roles of Src Family and JNK MAP Kinases in Signaling during Phagocytosis via αvβ5 Integrin and Mer Tyrosine Kinase Receptors.

G. Kanhai, S. Finnemann; Biology, Fordham University, Bronx, NY

Retinal Pigment Epithelial (RPE) cells are the most active phagocytes in the body as they are responsible for the diurnal uptake of photoreceptor outer segment (POS) fragments that shed in a circadian rhythm. Coordinated POS shedding and RPE phagocytosis maintain photoreceptor function and viability for life and breakdown in either process eventually causes blindness. Two RPE surface proteins, αvβ5 integrin and the tyrosine kinase receptor Mer (MerTK), are required for RPE phagocytosis, as lack of either in mutant mice impairs POS uptake and vision. In response to shed POS opsonized with the soluble integrin ligand glycoprotein MFG-E8, αvβ5 receptors stimulate a cytoplasmic signaling pathway in RPE cells in the eye and in culture. This pathway involves focal adhesion kinase (FAK) and is required for tyrosine phosphorylation and likely activation of MerTK, whose function is essential for engulfment of integrin-bound POS. Additional signaling proteins that may function to relay activation of αvβ5-FAK to MerTK and thus to link the distinct phagocytic steps of recognition and engulfment remain to be identified. We hypothesized that Src family and JNK MAP kinases may participate in the cytoplasmic signaling cascade during RPE phagocytosis in addition to FAK. We studied phagocytosis by RPE cells in culture fed with isolated porcine POS in the presence of the αvβ5 ligand MFG-E8. We found that POS-MFG-E8 challenge, but not incubation with MFG-E8 alone, caused short-term activation of both c-Src and JNK2. Furthermore, selective inhibition of either c-Src only (by dominant-negative c-Src), Src family kinases or JNK2 (using small-molecule inhibitors) in phagocytosis assays significantly diminished the ability of RPE to take up POS. Inhibition of c-Src moderately reduced POS binding and abolished POS engulfment. In contrast, inhibition of JNK2 had little effect on POS binding but robustly reduced engulfment. Our preliminary evidence suggests that c-Src may be downstream of JNK2. Ongoing experiments aim to elucidate the functional relationship between these two cytoplasmic kinases and MerTK in the RPE phagocytosis signaling mechanism.

100/B42

Laminin Binds to Myostatin and Modulates Its Activity.

N. Yasaka, J. Wakamatsu, A. Hattori, T. Nishimura; Meat Science Laboratory, Graduate School of Agriculture, Hokkaido University, Sapporo, Japan

Myostatin is a growth and differentiation factor belonging to the TGF-β superfamily, which acts as a negative regulator of skeletal muscle mass (McPherron et al., 1997). Although the mechanism whereby myostatin controls the proliferation and differentiation of myogenic cells is mostly clarified (Thomas et al., 2000), the regulation of myostatin activity after its secretion in the extracellular matrix (ECM) is still unclear. In the previous report, we have shown that decorin, a small leucine-rich proteoglycan, binds to myostatin, and that immobilized decorin in the collagen matrix prevents myostatin-mediated inhibitory action to myoblast proliferation (Miura et al., 2006). However, it is still unknown whether other ECM components than decorin could bind myostatin and modulate its activity. So, in the present study, we investigated the binding of laminin, one of major components of basement membrane, to myostatin and the effect of laminin on myostatin signaling. Surface plasmon resonance assay showed that laminin bound to mature myostatin with a dissociation constant (KD) of 2.30 × 10-8 M. Laminin also bound to Activin Receptor Type II B (ActRIIB), a receptor of myostatin, with a KD of 3.52 × 10-8 M. These results indicate that laminin binds both myostatin and its receptor. Next, we examined the action of laminin on the myostatin signaling pathway. Myostatin signaling was monitored by a conventional reporter assay in human A204 rhabdomyosarcoma cells. After transfection with CAGA-lux, which is a luciferase plasmid responsive to myostatin, cells were incubated with myostatin (10 ng/ml) and laminin (0-1000 ng/ml) for 24 hours. The luciferase activity was repressed significantly ($P<0.05$) by coincubation with laminin in a dose-dependent manner. These results suggest that laminin could bind to myostatin and/or ActRIIB and attenuate myostatin signaling. McPherron et al. (1997) Nature, 387, 83-90; Thomas et al. (2000) JBC, 275, 40235-40243; Miura et al., (2006) BBRC, 340, 675-680.

101/B43

Modulation of Myofibroblasts in the Rabbit Wound Healing Experimental Model.

C. El Kahi, B. Atiyeh, R. Jurjus, I. Abdallah, S. Dibo, A. Jurjus; AUB, Beirut, Lebanon

Introduction: Cutaneous wound healing is a complex process encompassing a number of overlapping phases, including inflammation, epithelialization, angiogenesis and matrix deposition whereby myofibroblasts are greatly involved. Wound contraction by means of the myofibroblast is a fundamental stage to reestablish the continuity of the wounded area; the latter is characterized by a contractile apparatus in its cytoskeleton especially with the presence of α-smooth actin (αSMA).Treatment with Moist Exposed Burn Ointment (MEBO) seems to enhance wound healing and results in less scar tissue. Purpose: first, to study the modulation of myofibroblasts in a full thickness wound performed on rabbits treated with different ointments. Second to examine the adhesion molecule, integrin, in granulation tissue. Methods: full thickness wounds were performed on 16 rabbits divided intoto four groups. Each group was treated with one of the four following treatments: 1-saline, 2-Tegaderm occlusive dressing 3-Silver Sulfadiazine 4-MEBO. Biopsies were taken at days 0, 4, 8,12,16,20 and processed for light microscopically and immunofluoresence to reveal αSMA and integrin αvβ3 expressions under the various treatments. Results: clinical immediate reepithelialization 4 days faster in the MEBO group compared to the other therapies with delayed initial contraction, earlier recruitment of myofibroblasts and Integrin αvβ3. Conclusion: Healing with MEBO showed earlier epithelialization and decreased activity of myofibroblasts despite the normal levels expression of αSMA. The data demonstrated the actual modulation of myofibroblasts by topical application of ointments.

102/B44

ATP Augments Osteoblastic Response to Mechanical Stretch by Modulating Cell Prestress.

J. Qi[1,2], L. Chi[1], J. Wang[1], R. Samanasinghe[1], M. Wall[1], M. Tsuzaki[1], A. J. Banes[1,2]; [1]Flexcell International Corporation, Hillsborough, NC, [2]Biomedical Engineering, University of North Carolina at Chapel Hill and North Carolina State University, Chapel Hill, NC

Introduction - It is broadly accepted that the intrinsic strain of a cell (also called prestress) affects cellular responses to mechanical stimulation. However, it is not agreed as to how changes in cellular prestress affect the transduction of mechanical stimuli into biochemical signals. We and other groups have reported that mechanical stress stimulates ATP release from the stimulated cells. Therefore, it was hypothesized that ATP may act as a modulator (+ or -) to regulate the degree of mechanosensitivity in cell. In the present study, mouse MC3T3-E1, osteoblast-like cells, were used as a model system. A mechanosensitive gene, Runx2, was used as a marker to monitor the cellular response to applied strain in 3D. Methods - MC3T3-E1 cells were grown in three-dimensional type I collagen gels to quiescence and subjected to a cyclic, uniaxial strain of 3.5% at 1 Hz for 6 h. The expression levels of Runx2 were determined with real-time RT-PCR. Results and Conclusions - The results showed that ATP reduced cell prestress by down-regulating the expression of matrix proteins and reorganizing cytoskeleton. Stretch up-regulated the expression of Runx2 by over 100% in the ATP-treated group compared to 20% in the control group. These results indicate that a decrease in cell prestress in this system increases cell mechanosensitivity to applied substrate strain. This is a controversial observation given that an accepted idea is that the greater the cell prestress the greater the cell mechanosensitivity. Further investigation with prestress-reducing chemicals, such as RGD-containing peptides to relieve integrin attachements and cytochalasin D to relieve the actin cytoskeleton will shed light on this observation.

103/B45

The Role of ALK1 and Endoglin Signaling in Scleroderma Fibrosis.

E. Morris[1], A. Bujor[1], P. ten Dijke[2], M. Trojanowska[1]; [1]Rheumatology and Immunology, Medical University of South Carolina, Charleston, SC, [2]Molecular Cell Biology, Leiden University Medical Center, Leiden, Netherlands

Objective: Scleroderma (SSc) is a disease of the connective tissues characterized by excessive production of extracellular matrix proteins (ECM). Transforming growth factor beta (TGF-β) is an inducer of ECM proteins in fibroblasts. Previous studies indicate that TGF-β signaling is activated in SSc via upregulation of the TGF-β type I receptor, ALK5, and pSmad1 (Pannu et al., JBC 2007 282(14)10405-10413, Arthr & Rheum 2008 58(8)2528-2537). In endothelial cells, TGF-β signals through two type I receptors and their interacting Smad proteins; ALK5 and pSmad2/3 or ALK1 and pSmad1/5/8. Expression of a type III receptor, endoglin, promotes signaling through Alk1. The goal of this study is to determine the role of endoglin and ALK1 signaling in the fibrotic phenotype of SSc fibroblasts. Methods: This study used adult SSc fibroblasts from skin biopsies and matched normal controls (NS). We measured mRNA levels of ALK5, ALK1, and endoglin using qRT-PCR. Protein expression of ALK5, ALK1, endoglin, collagen I, connective tissue growth factor (CCN2), pERK1/2, ERK1/2, pSmad1, and Smad1 was measured with western blotting. Endoglin expression was inhibited in NS and SSc fibroblasts using a siRNA adenovirus. Constitutively active ALK1 (caALK1) was overexpressed using adenovirus. Results: Data analysis showed that increased levels of ALK5 correlate with increased levels of endoglin in SSc fibroblasts. Downregulation of endoglin in NS fibroblasts led to an increase in ECM proteins. In contrast, downregulation of endoglin in SSc fibroblasts led to a decrease in ECM proteins suggesting that endoglin is a positive regulator of ECM in SSc. Consistent with this observation, overexpression of caALK1 in NS fibroblasts led to increased levels of pSmad1, activation of pERK1/2, and increased expression of collagen I and CCN2. Conclusions: Endoglin plays opposite roles in matrix gene regulation in NS and SSc fibroblasts. This data further supports the activation of ALK5/ALK1 signaling in SSc and demonstrates that endoglin is an important component of the TGF-β receptor complex. Overexpression of caALK1 in NS fibroblasts is sufficient to reproduce the main profibrotic features of SSc, including elevated ECM production.

104/B46

BMP4/Smad1 Signaling Is a Key Pathway in Diabetic Nephropathy.

T. Tominaga, H. Abe, A. Mima, T. Doi; Department of Clinical Biology and Medicine, University of Tokushima School of Medicine, Tokushima, Japan

Diabetic nephropathy is the most common cause of chronic kidney disease (CKD) and end-stage renal disease in the world. The pathogenesis of the renal lesions is characterized by thickening of glomerular basement membrane (GBM) and mesangial matrix expansion. Advanced glycation end-products (AGEs) accumulated as a result of hyperglycosemia induces production of extracellular matrix (ECM) in the process

of development in diabetic nephropathy. We have recently demonstrated that Smad1 regulates the expression of genes which constitute ECM. This study is to elucidate the role of BMP4/Smad1 signaling for development of diabetic nephropathy. Here we demonstrated that expression of BMP4 was induced by AGEs in vitro. The expression of BMP4 and Col4 were decreased by knockdown of receptor of AGEs (RAGE). In addition, the activation of Smad1 and the production of Col4 have increased by BMP4 treatment. We performed inhibition assay for Col4 using siRNA for BMP4 and the BMP4 neutralization antibody. The suppression of BMP4 inhibited the expression of Col4 in mesangial cells. Knockdown of BMP4 type Ia receptor (ALK3) has similarly decreased the expression of Col4. We examined that the role of BMP4 in diabetic nephropathy in vivo using transgenic mice carrying the mouse type 2-inducible nitric-oxide synthase (iNOS Tgm) while resembled human diabetic nephropathy. Although no expression of BMP4 and Smad1 were observed in normal glomeruli, these proteins were clearly expressed in sclerotic glomeruli on iNOS Tgm. Further direct evidence for BMP4 action in vivo was confirmed by using conditional knock-in transgenic mice for BMP4 (BMP4 Tgm). BMP4 Tgm did not cause diabetes, but showed significantly glomerulosclerosis similar to diabetic nephropathy. BMP4 Tgm also expressed the phosphorylated Smad1 and increased expression of Col4 in parallel with mesangial expansion.. These findings implicate that BMP4/Smad1 signaling pathway plays a critical role for the progression of diabetic nephropathy.

105/B47

Elastic Culture Models to Examine the Role of Tension-mediated Growth Factor Release in hMSC Lineage Specification.

R. M. Tenney, D. Discher; Molecular/Cell Biophysics lab, University of Pennsylvania, Philadelphia, PA

Human mesenchymal stem cells (hMSC) are being intensely studied because of their potential in therapeutic applications. hMSC differentiation into various lineages can be induced in vitro by various means, including the addition of growth factors and particularly TGFβ superfamily proteins. Our lab had previously demonstrated that matrix Elasticity can also direct hMSC lineage specification and differentiation. Stiffer substrates resulted in increased integrin adhesion as well as increased cytoskeletal organization and, consequently, intracellular tension. Wipff et al. recently reported a mechanical component to activated release of TGFβ by myofibroblast contraction against a resistant substrate (JCB 2007). TGFβ family proteins are synthesized as latent precursors, stored extracellularly, and are at times bound to the cell surface through integrins. We therefore hypothesized that increased hMSC intracellular tension coupled to matrix resistance could also release integrin-bound latent TGFβ on stiffer elastic substrates, thus providing key early differentiation signals. We have found that differentiation markers of the TGFβ family are induced in a substrate stiffness dependent manner. However, ratios of inactive to active TGFβ protein produced by hMSC differ while the levels of latency-associated protein remained relatively constant. We also show that manipulation of a latency-associated protein alters the expression pattern of lineage-specific markers. In conclusion, our results provide mechanistic evidence of an interplay between matrix/contractility/growth factor release in hMSC mechanosensitivity and differentiation.

106/B48

Cell Shape Regulates SRF-mediated Transcription to Control Proliferation.

M. Wozniak[1], C. Cheng[1], C. J. Shen[1], D. Pirone[2], C. S. Chen[1]; [1]Bioengineering, University of Pennsylvania, Philadelphia, PA, [2]Science, Mount St. Mary's University, Emmitsburg, MD

It has been known for some time that restricting cell shape decreases proliferation, although the mechanism by which this occurs is not well understood. Proliferation is regulated by changes in gene expression. However, it is unclear how changes in cell shape and adhesion mediate the transcriptional changes underlying proliferative control. We hypothesized that specific transcription factors are regulated by changes in cell shape. To test this, we analyzed microarray data obtained from cells in which the degree of cell spreading was manipulated using micropatterned islands of fibronectin. Several transcription factors with significant expression changes or with significant binding site overabundance in the gene promoters were identified, one of which was serum response factor (SRF). Real time PCR and luciferase assays revealed that unspread cells decreased expression of SRF-target immediate early genes when the promoter contained the full serum response element (SRE), which includes two promoter elements--the CArG box (binds SRF) and the Ets site (binds cofactors). However, genes in which promoters lack the Ets site exhibited no change in activity in unspread versus spread cells, suggesting that cell shape changes do not indiscriminately affect all SRF-dependent promoters. Rather, these data suggest that the Ets site in the SRE confers shape-dependent regulation of SRF. Because map kinases (MAPKs) are known to regulate SRF cofactor binding, the roles of different MAPK pathways responsible for mediating shape-regulated SRF activity and proliferation were examined. Unexpectedly, ERK was not required for SRF activity. Remarkably, inhibition of p38 MAPK rescued both SRE luciferase activity and proliferation in unspread cells, while inhibition of JNK decreased luciferase activity and proliferation (to levels of unspread cells). We are currently characterizing which SRF cofactors are downstream of JNK and p38 to determine, mechanistically, how cell shape regulates immediate early gene expression to control proliferation. These data highlight the pivotal role that cell shape plays in transcriptional regulation and proliferation and points to a novel mechanism by which this occurs.

107/B49

C-terminal Region-induced Structural Integrity of the Eighth Reelin Repeat Reflects the Signaling Activity of Reelin.

T. Kohno[1], Y. Nakano[1], N. Kitoh[1], H. Yagi[2], K. Kato[2,3], A. Baba[1], M. Hattori[1]; [1]Biomedical Science, Nagoya City University, Nagoya, Japan, [2]Structural Biology and Biomolecular Engineering, Nagoya City University, Nagoya, Japan, [3]Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki, Japan

Reelin is a secreted glycoprotein that plays pivotal roles in the development and function of the mammalian brain. Despite intensive research, how it activates downstream intracellular signaling is not fully understood. We recently found that the highly conserved C-terminal region (CTR) of Reelin is required for its full signaling activity; however, the underlying mechanism remains unknown. During biochemical study of Reelin, we serendipitously found that one commercially available anti-Reelin antibody G20 can bind CTR-lacking mutant Reelin proteins, but not wild-type Reelin, in western blotting. The G20 epitope resides in the last 19 residues of Reelin-repeat 8 (RR8), and neither posttranslational modification nor proteolysis can explain this effect. Furthermore, when an unrelated sequence, such as FLAG-tag, is inserted between RR8 and CTR, reactivity of the corresponding antibody decreased greatly, suggesting that RR8 and CTR form a tight

structure that makes the surrounding sequence inaccessible to an antibody. Taking advantage of this phenomenon, we show the existence of the CTR-lacking Reelin isoform in vivo for the first time, and estimate its contribution to the total amount of secreted Reelin. Importantly, the extent to which Reelin mutants react with G20 is inversely correlated with their signaling activity, indicating that the CTR-induced structural integrity of RR8 is a prerequisite for downstream signaling activation. The present study also raises a caveat that immunoreactivity, even on western blotting, can vary depending on its surrounding structure, and that the inserted epitope tag activity can be affected.

108/B50

Excitability of Dictyostelium discoideum Is Regulated by the Ratio of Membrane Bound to Secreted Phosphodiesterase.

E. Palsson; Biology, Simon Fraser University, Burnaby, BC, Canada

After onset of starvation randomly distributed *Dictyostelium discoideum* cells initiate formation of cooperative aggregation territories over a wide range of initial cells densities. Recruitment of a high number of cells is important, and it appears that formation of large territories has been selected for. Propagating cAMP waves enable the formation of territories larger than 1 cm diameter. These cAMP waves are generated by the means of an elaborate cAMP signaling system that makes the whole field of cells excitable. *Dictyostelium* cells respond chemotactically to these waves, guiding cell aggregation towards a signaling center. An important component of the signaling system is the PdsA phosphodiesterase (PDE) that breaks down the external cAMP. PdsA can be either membrane bound or secreted. Here I show that by utilizing both forms of PDE and by fine tuning the ratio, *Dictyostelium* extends the range of cell densities where cAMP waves can propagate and thus where aggregation can be successful. The membrane bound PDE reduces the likelihood that the aggregation territory breaks up into many smaller territories, as the cell density increases, while the secreted PDE is important for wave propagation at low cell densities. An interesting interpretation of these findings is that for some general excitable systems, with discrete point sources located far apart, wave propagation is not possible if the sink is in the same location as the source. However, when the source and sink are in a different location, wave propagation is possible. One example is Ca^{++} propagation in cardiac cells .

109/B51

Regulation of p38 MAP Kinase by Anastellin Does Not Require Formation of Superfibronectin.

R. You, R. Klein, M. Zheng, P. J. McKeown-Longo; Center for Cell Biology and Cancer Research, Albany Medical College, Albany, NY

Anastellin is a peptide derived from the first Type III module (III1c) of fibronectin (Fn). Anastellin has been shown to inhibit tumor angiogenesis and metastasis in mouse models of human cancer. In addition, anastellin has been shown to stimulate the formation of a polymerized form of Fn, termed superfibronectin (sFn), and to regulate map kinase (MAPK) signaling pathways. However, the molecular basis underlying the anti-tumor activity of anastellin remains poorly understood. The present study was undertaken to determine whether there is any cause-effect relationship between the ability of anastellin to form sFn and to activate p38 MAPK in human dermal fibroblasts. Cells were treated with recombinant His-tagged wild-type anastellin or mutant anastellin (L37A/Y40A). The anastellin mutant was unable to form sFn when tested in Fn polymerization assays. Both forms of anastellin, but not control Fn Type III modules, bound equally well to Fn in the matrix and increased the phosphorylation of p38 MAPK in dermal fibroblasts. Activation of p38 by both forms of anastellin was dose-dependent and occurred within minutes. Both anastellin and the anastellin mutant induced p38 activation in mouse embryo fibroblasts null for Fn, indicating that there was no requirement for Fn in the activation of p38 by anastellin. Adding back Fn to Fn null fibroblasts did not alter either the kinetics or extent of p38 activation by anastellin, indicating that the activation of p38 MAPK by anastellin was not modulated by the presence of Fn. Anastellin has also been reported to support β1 integrin dependent cell adhesion. However, further studies indicated that activation of p38 by anastellin did not require either cell adhesion or β1 integrin, as anastellin activated p38 in both suspended cells and in cells null for β1 integrin. These data indicate that the activation of p38 by anastellin does not depend on the formation of sFn. They further suggest that p38 activation by anastellin is independent of fibronectin and β1 integrins. Our findings are consistent with a model whereby anastellin action requires direct binding to cell surface receptor(s) causing the activation of p38 MAPK. This work is supported by NIH grant CA69612.

110/B52

A Novel Role for RGS-GEFs in Fibronectin-induced RhoA Activation.

A. D. Dubash, K. Wennerberg, R. Garcia-Mata, M. Menold, K. Burridge; Cell and Devt Bio, UNC Chapel Hill, Carrboro, NC

Adhesion of cells to extracellular matrix proteins such as fibronectin triggers a wide range of different signaling cascades important for the growth and survival of cells. The ability of cells to attach to, spread and migrate on fibronectin is controlled by dynamic rearrangements of the actin cytoskeleton, a process that is mainly controlled by members of the Rho family of GTPases such as Cdc42, Rac1 and RhoA. While significant advances have been made in understanding how Rho proteins control the cytoskeletal architecture, less is known about the signals controlling activation of the GTPases themselves. The focus of this study was to determine which guanine nucleotide exchange factor(s) are responsible for activation of RhoA downstream of fibronectin adhesion. Using an affinity pulldown for activated exchange factors, we show that the RhoA-specific exchange factors Lsc/p115 RhoGEF and LARG are activated when cells are plated onto fibronectin. This effect is specific to Lsc/p115 RhoGEF and LARG, as other exchange factors such as Ect2 or Dbl are not activated by FN. Knockdown of Lsc and LARG together significantly decreases RhoA activation, stress fiber and focal adhesion formation downstream of fibronectin adhesion. Identical results on RhoA signaling are obtained with a DH-dead mutant of p115 RhoGEF, suggesting that catalytic activity of the GEF is necessary for downstream signaling to RhoA. p115 RhoGEF and LARG are part of a family of RhoA GEFs known as RGS-GEFs, which are traditionally known for their involvement in LPA-induced RhoA activation. Interestingly, we observe that GPCRs are not involved in FN-induced p115 RhoGEF activation, and that integrin adhesion is sufficient to activate p115 RhoGEF. These data therefore establish a previously uncharacterized role for the exchange factors Lsc/p115 RhoGEF and LARG in linking fibronectin signals to downstream RhoA activation.

111/B53

Fibronectin Dependent Endothelin-1 Synthesis in Rat Hepatic Myofibroblasts via a Src/ERK Signaling Pathway.

S. Zhan, D. Rockey; University at Texas Southwestern, Dallas, TX

Liver injury is characterized by activation of stellate cells, a process that leads to enhanced synthesis of various cytokines and peptides, including the vasoactive peptide, endothelin-1. In turn, endothelin-1 has autocrine effects on stellate cells, and induces their cellular contraction. We hypothesized that the extracellular matrix (ECM) signals to endothelin-1 synthetic pathways with resultant production of endothelin-1. Here we demonstrate that the ECM component, fibronectin leads to significant increases in endothelin-1 production. As predicted, the mechanism was linked to engagement of typical fibronectin integrins. However, after integrin binding, fibronectin led to enhanced synthesis of precursor endothelin-1 rather than production of endothelin converting enzyme. Further, we defined a signaling pathway proceeding from fibronectin through Src, Shc, and ERK to preproendothelin-1 transcription and synthesis. Fibronectin's stimulation of endothelin-1 in stellate cells also had important functional consequences; we demonstrated in a fibronectin/type I collagen gel contraction assay that fibronectin mediated induction of endothelin-1 synthesis specifically led to increases in stellate cell contraction. Finally, we demonstrated that stellate cells activated in vivo respond to fibronectin by stimulation of endothelin-1 synthesis. The data indicate that fibronectin stimulates endothelin-1 synthesis by stellate cells via a specific signaling pathway, with resultant autocrine functional effects that are likely to be important in vivo during the wound healing process.

112/B54

Fibronectin Growth Factor-binding Domains Are Required for Fibroblast Survival and Growth in Response to PDGF-BB.

R. A. Clark[1,2], F. Lin[1], X. Ren[2,3], L. Macri[1], Z. Pan[4], M. Tonnesen[2], M. Rafailovich[4], W. Zong[5], D. Mosher[6], D. Bar-Sagi[7]; [1]Biomedical Engineering, Stony Brook University, Stony Brook, NY, [2]Dermatology, Stony Brook University, Stony Brook, NY, [3]Pharmacology, Stony Brook University, Stony Brook, NY, [4]Materials Science, Stony Brook University, Stony Brook, NY, [5]Molecular Genetics, Stony Brook University, Stony Brook, NY, [6]Biomolecular Chemistry, University of Wisconsin, Madison, WI, [7]Biochemistry, New York University, New York, NY

Fibroblast ingrowth is the rate-limiting step in wound healing and appears to require fibronectin (FN), which is absent in chronic wounds and burns. Failure of chronic wounds to heal and progression of burn injury are associated with diminished fibroblast growth and survival. We have found that FN contains 4 growth factor-binding (FN-GFB) sites for PDGF that are required for fibroblast survival and proliferation. The 4 FN-GFB sites are homologous and each binds PDGF with a KD of ~10-100 nM. To determine mechanisms by which fibroblasts fail to survive and grow in the absence of FN-GFB sites, FN-null fibroblasts were utilized. Null- cells cultured on recombinant RGD cell-binding domains without a FN-GFB site died by apoptosis within three days, in the presence or absence of PDGF-BB. Attachment, spreading and focal contact formation occurred within 1 hr and appeared morphologically normal for 24 hrs. However, metabolism, even in the presence of PDGF, was minimal at 24 hrs and was associated with autophagy, as judged by the appearance of LC3-II. At 48 hrs apoptosis was first noted, and by 72 hrs most cells had died. In contrast, FN-null cells plated on any FN-GFB site in contiguous or noncontiguous arrays with RGD cell-binding domain survived for 7 days without PDGF and proliferated in its presence. In the absence of FN-GFB sites, PDGF-receptor-β was suboptimally phosphorylated in response to PDGF, yet Akt,TSC, ERK1/2, p38 and JNK phosphorylation appeared normal, while phosphorylation of S6 kinase1 and ribosomal protein S6 were remarkably decreased. These data are consonant with downstream dysregulation of the mTOR pathway. Thus, in the absence of FN-GFB sites, cells developed a starvation response with the induction of autophagy, and finally died by apoptosis. These FN-GFB sites may have important therapeutic for FN-deficient wounds, such as burns and chronic ulcers.

Cadherins (113-123)

113/B55

Impaired E-cadherin-dependent Cell-Cell Adhesion by MMP13.

B. Bai[1,2], C. Li[1], C. Jamora[1]; [1]Biology Department, University of California, San Diego, La Jolla, CA, [2]Dermatology Department, The Second Affiliated Hospital of Harbin Medical University, Harbin, China

E-cadherin mediated intercellular adhesion is essential for the maintenance of the organization and integrity of the epidermis. The soluble ectodomain of E-cadherin has been observed in the serum of many inflammatory skin diseases and cancer. Several proteases have been implicated in the ectodomain cleavage of E-cadherin and this cleavage was interfered by tissue inhibitor of metalloproteinases (TIMPs) which incriminates the matrix metalloproteinase (MMP) family of proteins in E-cadherin shedding. Here we demonstrated that MMP 13, an enzyme associated with carcinogenesis, is capable of E-cadherin shedding in mouse and human keratinocytes. Overexpression of MMP 13 resulted in impaired E-cadherin-dependent cell-cell adhesion and altered cellular morphology. This study identified E-cadherin as a new substrate for MMP13, which can impact basic cellular process such as cell migration and invasion, as well as induction of blister formation and inflammatory cell infiltration in diseases including eczema and psoriasis.

114/B56

Vinculin Regulates Cell Surface Expression of E-cadherin.

X. Peng, K. DeMali; University of Iowa, Iowa City, IA

E-cadherin, the major cell surface receptor present in the adherens junctions, is critical for initiating and maintaining cell adhesion. E-cadherin is tightly regulated at the level of synthesis, trafficking to and from the plasma membrane, and by the strength by which it is bound to cadherins on neighboring cells. These properties of E-cadherin are modulated by proteins that are recruited to its cytoplasmic domain. Deletion studies in mice or cells show that vinculin, an actin binding protein, is critical for the organization of cell-cell junctions. However, gene deletion approaches inhibit vinculin expression at both sites of cell-cell and cell-matrix adhesion, making it impossible to distinguish the contribution of vinculin at each site. Furthermore, over-expressed vinculin does not readily integrate into cell-cell junctions. As a result, the

function of vinculin in adherens junctions has remained elusive. To define the role of vinculin at sites of cell-cell adhesion, we have generated a powerful vinculin short hairpin RNA knockdown/substitution model system that perturbs vinculin specifically at cell-cell adhesion. We found that decreased vinculin expression causes a loss of E-cadherin localization to the adherens junctions, a loss of the honeycomb-like shape characteristic to epithelial cells, and decreased adhesion to cadherin extracellular domains. We examined if the observed effect was due to a failure of E-cadherin to localize to the junction during assembly or an inability to be maintained at this site using a Ca++ switch assay. We found that cells with decreased vinculin expression are impaired in junctional assembly. The mechanism for the effects of vinculin will be addressed. Taken together these results suggest a novel function for vinculin in cell-cell junctions, namely that vinculin regulates cell surface expression of E-cadherin.

115/B57

E-cadherin Residue Lys738 and p120-α-catenin Interaction Are Two Independent Regulators of E-cadherin Adhesion.

S. M. Troyanovsky[1], O. Laur[2], S. Hong[1], R. Troyanovsky[1]; [1]Dermatology, Northwestern University, Chicago, IL, [2]Emory University Medical School, Atlanta, GA

While it has been shown that intracellular cadherin regions containing catenin-binding sites are crucial for intercellular adhesion, the exact mechanisms of how catenins regulate cadherin adhesion activity are not completely understood. To evaluate these mechanisms, we studied adhesion defects of two cadherin deletion mutants Ec1Δ(748-882) M lacking both the p120- and β-catenin-binding sites and Ec1Δ(812-882)M only lacking the β-catenin-binding site. We showed that postanslational modification, apparently ubiquitination of the membrane-proximal conserved cadherin residue Lys738 is responsible for high instability of the cadherin mutant Ec1Δ(748-882)M. Substitution of this residue with Arg dramatically increased the lifetime of this mutant and completely restored its recruitment into the cell-cell junctions. Furthermore, such a mutant became functional in cadherin-deficient CHO cells or in A-431 cells depleted for α-catenin. K738R replacement, however, was unable to rescue the adhesion potential of the mutant Ec1Δ(812-882)M containing the intact p120-binding site. Surprisingly, the high instability of this mutant appeared to be caused by its weak binding to p120. Overexpression of p120 or just p120 arm domain completely restored the recruitment of this mutant into the adherens junctions. We also showed that the lack of α-catenin in the cadherin-catenin complex was responsible for the low affinity of p120-cadherin interactions. Our data suggest that posttranslational modification of Lys737 or destabilization of α-catenin-p120 interactions by various signaling systems could be independent regulators of cadherin adhesion.

116/B58

Endocytosis Is Required for E-cadherin Redistribution at Mature Junctions.

S. de Beco[1], C. Gueudry[1], F. Amblard[1,2], S. Coscoy[1,2]; [1]UMR 168, Institut Curie, Paris, France, [2]CNRS, Paris, France

In mature epithelium, cells occupy relatively stable positions with respect to each other. This implies that the molecular framework producing adhesive forces is precisely adjusted to be mechanically equivalent on both sides of all junctions. To study the molecular mechanisms underlying mechanical homeostasis, we postulated that stability is maintained thanks to a permanent correction of forces unbalance, achieved through a dynamical adjustment of molecular components on both sides of cell-cell junctions. The mechanisms controlling E-cadherin surface density at junctions remain poorly understood. We thus investigated the turnover dynamics of E-cadherin. We used two-photon Fluorescence Photobleaching combined with fast 3D wide-field imaging to spatially analyze fluorescence recovery. We show that most E-cadherins undergo no lateral diffusion along junctions, but that their turn-over is rate-limited by endocytosis. Indeed, in confluent cultures of MCF7 or MDCK cells, E-cadherin-GFP is rapidly recycled, and no lateral mobility is observed when endocytosis is pharmacologically blocked. This suggests that endocytosis directly acts upon immobilized cadherins, and raises important questions about the endocytosis of engaged adhesion receptors. The fact that endocytosis is the rate-limiting process for regulating cadherin levels and possibly adhesive junction strength, leads to new questions on the mechanical aspects of endocytosis and its regulation local scale. Moreover, we recently observed that a perturbation of mechanical equilibrium by Hepatocyte Growth Factor is correlates with an increased E-Cadherin turnover. Ongoing experiments aim at characterizing this dysplastic-like loss of stability. Furthermore, tissue stability being a collective phenomenon, we try to address the problem of instability propagation. We are thus currently studying the dynamic behavior of a junction between a "dysplastic-like" cell and "normal" neighbours. We study those normal/abnormal junctions using a "2-colour" FRAP method. This work should lead to understand the rules of mutual de/stabilization and disorder propagation for a single cell perturbation in a tissue.

117/B59

Identification of Distinct Regions within the Unique Cytoplasmic Domain of the Desmosomal Cadherin, Desmoglein 2, Which Regulate Endocytic Trafficking in Epithelial Cells.

J. Chen[1], B. V. Desai[1], J. L. Klessner[1], K. J. Green[1,2]; [1]Pathology, Northwestern Univ., Chicago, IL, [2]Dermatology, Northwestern University, Chicago, IL

Desmogleins (Dsgs) are transmembrane cadherins localized in desmosomes, intercellular junctions critical for maintaining mechanical integrity of epithelial tissues. We previously identified epidermal growth factor receptor (EGFR) as a regulator of cell surface Dsg2 in oral squamous cell carcinoma cells (OSCC), and more recently demonstrated that inhibition of EGFR interferes with Dsg2 internalization and increases intercellular adhesion. To identify sequences in the Dsg2 cytoplasmic tail that contribute to endocytic trafficking, we generated a panel of C-terminally FLAG-tagged Dsg2 wild type and mutants with intact ecto- and transmembrane domains, harboring successive truncations of the extended 500 amino acid tail, which includes an intracellular anchor (IA) and intracellular catenin binding segment (ICS), as well as unique downstream proline-rich linker (PL), repeating unit domain (RUD), and desmoglein terminal domains (DTD). Biotinylation experiments showed that all of the mutants can be delivered to the cell surface of retrovirally transduced OSCC. However, they were internalized with different kinetics. The mutant comprising the IA and ICS domain was internalized most efficiently: in reduced calcium (0.09mM), 70% of surface-labeled protein was internalized within 5mins and almost 100% within 30mins, compared with only ~45% of full length Dsg2 at this latter time point. Addition of the PL domain decreased internalization to a level comparable with full length Dsg2. Although addition of the RUD and DTD regions did not further attenuate internalization at 0.09mM calcium, an inverse correlation between Dsg tail length and the size of internalized pool was observed at 0.25mM calcium, at which level desmosomes begin to form. Intriguingly,

internalization of the mutants lacking RUDIV and V was not blocked by an EGFR inhibitor, suggesting that sequences in these regions confer sensitivity to EGFR inhibition. Collectively, this work identifies, for the first time, distinct functions for the Dsg2 cytoplasmic domain in regulating both constitutive and EGFR-dependent endocytic trafficking of this desmosomal cadherin.

118/B60

T-cadherin Localizes to the Centrosome and the Nucleus.

A. Andreeva[1], M. Kutuzov[1], I. Vardya[1], A. Krbanjevic[1], E. Talovskaya[2], V. Tkachuk[2], T. Voyno-Yasenetskaya[1]; [1]Pharmacology, University of Illinois at Chicago, Chicago, IL, [2]Cardiology Research Center, Moscow, Russian Federation

T-cadherin (H-cadherin, cadherin 13) is endogenously expressed in human vasculature in endothelial cells, smooth muscle cells, pericytes and cardyomyocytes. It is upregulated in vascular proliferative disorders and in tumor associated neovascularization, yet downregulated in a number of cancers. Unlike classical cadherins, T-cadherin lacks transmembrane and intracellular domains and is attached to the plasma membrane via GPI anchor. It lacks a number of amino acid residues crucial for adhesion and is mainly monomeric. Thus, T-cadherin may function rather as a signaling molecule than as adhesion mediator. Indeed, the involvement of T-cadherin in the cell cycle and in protection from apoptosis has been documented. However, it is not yet clear how T-cadherin may signal from the outer side of the plasma membrane to the cytoplasm and the nucleus. Although some interactive partners of T-cadherin at the plasma membrane have been recently identified, another possibility is that T-cadherin may also function at cellular locations other than the plasma membrane. To assess this hypothesis, we examined T-cadherin location in human pulmonary artery endothelial cells (HPAECs) and in umbilical vein endothelial cells (HUVECs) using confocal microscopy with two different T-cadherin antibodies. We found that a considerable proportion of T-cadherin is located in the nucleus and in the centrosomes. The nuclear location was also confirmed by immunoblotting upon cell fractionation. T-cadherin colocalized with several centrosomal markers throughout the cell cycle in HUVECs and in prophase and metaphase in HPAECs. In the telophase, T-cadherin was transiently concentrated in the midbody and apparently degraded. At least one of the T-cadherin-positive bands could be detected in gamma-tubulin immunoprecipitates. Overexpression of T-cadherin resulted in an increase in the number of multinuclear cells, while its downregulation by siRNA led to an increase in the number of cells with multiple centrosomes. These findings indicate that T-cadherin is a nuclear and centrosomal protein and that its deregulation in endothelial cells may lead to profound defects in the cell cycle via disturbance in cytokinesis or centrosomal replication.

119/B61

p120-Catenin Inhibits VE-cadherin Internalization through a Rho-Independent Mechanism.

C. Chiasson[1,2], K. B. Wittich[2], P. A. Vincent[4], V. Faundez[2], A. Kowalczyk[2,3]; [1]Graduate Program in Biochemistry, Cell, and Developmental Biology, Emory University, Atlanta, GA, [2]Department of Cell Biology, Emory University, Atlanta, GA, [3]Department of Dermatology, Emory University, Atlanta, GA, [4]Center for Cardiovascular Sciences, Albany Medical College, Albany, NY

p120-catenin (p120) is a cytoplasmic binding partner of cadherins and regulates cadherin cell surface levels by preventing cadherin endocytosis. p120 is also a potent inhibitor of the small GTPase RhoA, but the relationship between these functions of p120 is not understood. To define the mechanism by which p120 regulates VE-cadherin internalization, we first investigated the endocytic machinery responsible for processing VE-cadherin. We found that VE-cadherin is internalized via a mechanism that requires clathrin, AP-2, and dynamin. Chemical cross-linking and co-immunoprecipitation experiments further demonstrated that the VE-cadherin cytoplasmic tail specifically associates with clathrin and AP-2. Importantly, p120 prevents VE-cadherin from being recruited into membrane domains containing clathrin and AP-2. To determine if p120's function in stabilizing cadherins at the plasma membrane is mechanistically dependent upon its role as a Rho inhibitor, we used several approaches to inhibit RhoA activity. Interestingly, we found that inhibition of RhoA was unable to phenocopy p120 inhibition of cadherin internalization. Furthermore, p120 mutants that are deficient in RhoA inhibition retained the ability to efficiently block cadherin internalization, and these mutants prevented recruitment of cadherin into clathrin and AP-2 enriched membrane domains. We conclude that p120 regulates clathrin dependent endocytosis of VE-cadherin in a Rho-independent manner. These findings suggest a model in which p120 functions as a plasma membrane retention signal for VE-cadherin by preventing the recruitment of VE-cadherin into membrane domains enriched in components of the endocytic machinery.

120/B62

Involvement of M-cadherin, Rac1 and ARF6 Signaling Pathways during Myoblast Fusion.

S. Charrasse, F. Comunale, C. Gauthier-Rouvière; CNRS, Montpellier, France

Myoblast fusion results from an ordered sequence of events, including cell migration, alignment, recognition, adhesion and membrane merging but the regulatory circuits are poorly known. In mammalian cells, molecules from the cadherin family are involved in cell-cell recognition and adhesion and they act as adhesion-activated signaling receptors which regulate Rho family GTPases. Rho GTPases promote the reorganization of the actin cytoskeleton, an essential process during myoblast cell fusion. Recently, we have showed that M-cadherin-dependent adhesion activates the Rho GTPase Rac1 via the Rho-GEF Trio at the time of myoblast fusion. Cadherins form a large complex at the plasma membrane, and by co-immunoprecipitation experiments using C2C12 myoblasts, we found that ARF6 GTPase is associated with M-cadherin and Rac1 at the fusion time. During the differentiation process of C2C12 myoblast, the ARF6 GTPase is activated just prior Rac1, an event required for myoblast fusion. ARF6 knockdown decreases myotube formation and modifies myoblast morphology. Analysis of myotube morphology and number and measurement of the fusion index after transfection of Rac1 and/or ARF6 in their different activation states (WT, DN, CA) confirms that both GTPases are involved in myotube formation. Morever ARF6 and Rac1 activities were both detected during skeletal muscle regeneration. In conclusion we show that ARF6 participates in myoblast fusion into myotubes in combination with Rac1.

121/B63

N-cadherin/120 Catenin Association at Cell-Cell Contacts Occurs in Lipid Rafts and Is Required for RhoA Activation and

Myogenesis Induction.

N. Taulet, F. Comunale, S. Charrasse, C. Favard, S. Bodin, C. Gauthier-Rouvière; CRBM, CNRS, Montpellier, France

N-cadherin, a homophilic cell-cell adhesion molecule, is implicated in the commitment to myogenesis. In C2C12 myoblasts N-cadherin at the cell-cell contacts acts as an adhesion-activated receptor which activates RhoA GTPase, an event required for myogenesis induction. Here we show that the association of p120 catenin with N-cadherin at the cell-cell contacts occurs in membrane microdomains called lipid rafts. Moreover, we demonstrate that the interaction of p120 catenin with N-cadherin is required for the association of N-cadherin with lipid rafts and for its stabilization at cell-cell contacts enabling the formation of a functional adhesive complex. Disruption of lipid rafts leads to the inhibition of myogenesis induction and N-cadherin-dependent RhoA activation as does the perturbation of the N-cadherin/p120 catenin complex after the knockdown of p120 catenin. Finally, we show that RhoA GTPase is recruited to the N-cadherin complex present in lipid rafts. We propose that the formation of a functional N-cadherin/catenins complex occurs in lipid rafts and allows the recruitment of RhoA and the regulation of its activity during myogenesis induction.

122/B64

Differential Expression of Cadherin 23 Alternate Transcripts and Protein Isoforms in the Mouse and Primate Inner Ear and Retina.

A. Lagziel[1], N. Overlack[2], U. Wolfrum[2], S. L. Bernstein[3], R. J. Morell[1], T. B. Friedman[1]; [1]Laboratory of Molecular Genetics, National Institute on Deafness and Other Communication Disorders National Institutes of Health, Rockville, MD, [2]Institute of Zoology Cell and Matrix Biology, Johannes Gutenberg University of Mainz, Mainz, Germany, [3]Department of Ophthalmology & Visual Sciences, University of Maryland, Baltimore, MD

In humans, mutations of *CDH23* cause Usher syndrome type 1 D (USH1D), characterized by profound congenital deafness, vestibular areflexia and retinitis pigmentosa (RP) or nonsyndromic deafness DFNB12. The *waltzer (v)* mouse phenotype is due to mutations in *Cdh23* and is characterized by deafness and vestibular dysfunction, but not RP. We performed an analysis of cadherin 23 mRNA and protein variants expressed in the inner ears and retinae of wild type and mutant mice. Semiquantitative real-time PCR of *Cdh23* alternative splice isoforms in mRNA from wild type and homozygous v^{6J} mice revealed that *Cdh23* splice variants have different expression patterns in the inner ear and retina. Some, but not all, of these patterns are affected by the v^{6J} nonsense mutation. Immunohistochemistry, immunohistology, and EM analyses show that the cadherin 23_3 isoform, which lacks the extracellular and transmembrane domains, localizes exclusively to the basal body in the outer plexiform layer of the retina and to the synaptic region of auditory and vestibular hair cells in both mutant and wild-type mice. Western blot analyses of mouse inner ear and retina revealed that the longest *Cdh23* isoform (cadherin 23_1) is the only one affected by the v^{6J} mutation. Cadherin 23_1 is transiently expressed from embryonic age E13.5 to approximately postnatal day 12 in the wild-type mouse inner ear, but is not detected in the retinae of embryonic, juvenile or adult wild-type mice. Western blots of monkey and human retinae, however, do show expression of the cadherin 23_1 isoform. We hypothesize that the lack of retinal expression of the longest cadherin 23 isoform accounts for the lack of an RP phenotype in *waltzer* mice, and that smaller cadherin 23 isoforms are localized to regions previously not described.

123/B65

E-Cadherin Expression in Subconfluent and Confluent Fibroblast Cells.

R. O. Adekunle, J. Arpino, D. Hutter-Lobo; Monmouth University, Long Branch, NJ

E-cadherin, found mostly in epithelial tissue, is an integral protein that plays an important role in cellular adhesion. Loss of E-cadherin through down regulation decreases the ability of the cell to adhere, which results in an increase of cellular motility. Deficient E-cadherin function has been associated with cancer progression. Since the cell's ability to adhere has been reduced, the cancer cells are able to metastasize. E-cadherin has an 80 kDa unit that is located in the extracellular space. It has been confirmed that if the 80 kDa is cleaved, E-cadherin is no longer active. It has been observed that as the level of MMP-9 increases, a peptidase enzyme that plays a role in the degradation of the extracellular matrix, the level of E-cadherin expressed decreases. It has also been observed that E-cadherin stimulates the mitogen-activated protein kinase (MAPK) pathway, specifically the ERK MAP kinase. Therefore, as the expression of E-cadherin increases, so does the expression of ERK. Since previous results in our laboratory have shown that ERK expression is decreased in confluent cells, it was hypothesized that upstream signaling pathways may inactivate this expression, including cadherin-regulated signaling. Fibroblast cells (BJ) were used to determine whether E-cadherin was expressed differently in confluent and subconfluent cells, and also if E-cadherin expression was correlated to MMP-9 and ERK expression. Our results demonstrate that the inactive form of E-cadherin (80 kDa) is definitely expressed in normal fibroblasts (BJ cells), and is expressed at a slightly higher level in subconfluent cells than confluent cells. MMP-9, is also expressed in subconfluent BJ cells. These results suggest a possible regulation of E-cadherin-dependent signaling upon confluence. Expression of these proteins will also be compared in fibrosarcoma cells, which lack contact inhibition.

Cell-Cell Adherens Junctions (124-142)

124/B66

Unraveling the Effect of IFN-γ and TNFα on the Expression of Junctional Adhesion Molecule-C (JAM-C) in Mouse Germ Cells.

T. Leung, W. Lee, W. Lui; School of Biological Sciences, The University of Hong Kong, Hong Kong, China

Junctional adhesion molecule-C (JAM-C) was found expressed by germ cells and localized at the apical ectoplasmic specialization in seminiferous epithelium. Recent studies have shown that a spectrum of cytokines play crucial roles in regulating junction dynamics in the testis. In the present study, we sought to investigate the effect of interferon-γ (IFN-γ) and tumor necrosis factor α (TNFα) on the expression of JAM-C in testis. Addition of IFN-γ (5 ng/ml) and TNFα (10 ng/ml) (I+T) to mouse germ cell cell line GC2-spd(ts) reduced JAM-C mRNA and protein levels synergistically. Luciferase reporter assays have shown that I+T exerted a negative effect on JAM-C promoter. By site-directed mutagenic studies, a putative Sp1 motif located between nucleotides -68 and -56 in the promoter region was found to be responsible for the I+T- mediated JAM-C repression. Electrophoretic mobility shift assays (EMSAs) showed that Sp1, Sp3 and KLF4 were bound onto

the Sp1 motif and the binding of Sp1, Sp3 and KLF4 onto the Sp1 motif was significantly reduced upon I+T treatment. Western blotting has shown that, the level of Sp1, Sp3 and KLF4 in nuclear fraction were reduced in the presence of I+T. Taken together, it is postulated that Sp1, Sp3 and KLF4 form a ternary complex mediating JAM-C basal transcription and I+T decreases the binding of the ternary complex onto the promoter leading to gene repression. This work was supported by Hong Kong Research Grant Council (HKU771507M) and CRCG Seed Funding for Basic Research.

125/B67

Successive Post-translational Modifications of E-cadherin Are Required for InlA-mediated Internalisation of Listeria Monocytogenes.

M. Bonazzi, E. Veiga, J. Pizarro-Cerdá, P. Cossart; BCI, Institut Pasteur, Paris, France

Listeria monocytogenes surface proteins internalin (InlA) and InlB interact with the junctional protein E-cadherin and the HGF receptor Met respectively on the surface of epithelial cells to mediate bacterial entry. Here we show that InlA triggers two successive E-cadherin post-translational modifications, i.e. the Src-mediated tyrosine phosphorylation of E-cadherin followed by its ubiquitination by the ubiquitin-ligase Hakai. E-cadherin ubiquitination induces the recruitment of clathrin which is required for optimal bacterial internalisation. We also show that the initial clustering of E-cadherin at the bacterial entry site requires caveolin, a protein normally involved in clathrin-independent endocytosis. Strikingly clathrin and caveolin are also recruited at the site of entry of E-cadherin-coated sepharose beads and functional experiments demonstrate that these two proteins are required for beads entry. Together these results not only document how the endocytosis machinery is recruited and involved in the internalisation of a zippering bacterium but also strongly suggest a functional link between E-cadherin endocytosis and the formation of adherens junctions in epithelial cells.

126/B68

Dissection of α-catenin Organization and Function in Cells: II. Manipulation of Membrane-bound and Cytosolic Pools of α-catenin Reveal Functions of α-catenin in Regulating Actin and Membrane Dynamics.

J. Benjamin, W. Nelson; Biology, Stanford University, Stanford, CA

Alpha-catenin had been postulated to serve as a stable linker between the cadherin-catenin complex and the actin cytoskeleton. However, direct in vitro binding studies showed that α-catenin is an allosteric protein: monomer binds preferentially to β-catenin and homodimer binds preferentially actin and inhibits Arp2/3 activity. The new model posits that in the absence of cell-cell contact, when the concentration of the cadherin/catenin complex at the membrane is low, active Arp2/3-mediated actin polymerization drives lamellipodial protrusion. As cell-cell contacts are initiated and mature, the local concentration of the cadherin/catenin complex increases such that dissociated α-catenin can locally homodimerize in the cytosol, bind and bundle actin filaments and decrease Arp2/3 mediated actin polymerization. As a result, cadherin-mediated cell-cell adhesion is stabilized, and membrane activity and cell migration decreased. To test this model, we used the minimal α-catenin binding site on β-catenin to sequester the cytosolic pool of α-catenin to the plasma membrane (Lyn-βcat) or to mitochondria (ActA-βcat), thereby increasing or decreasing, respectively, the potential for α-catenin dimerization. Under these condition, increased plasma membrane-targeting of α-catenin in single cells led to decreased membrane activity independent of cell-cell adhesion. Conversely, mitochondrial-targeted α-catenin resulted in increased lamellipodial activity and increased rates of single-cell and cell monolayer migration. These effects were unexpected, since the cytoplasmic concentration of α-catenin in single cells is not predicted to effect Arp2/3-mediated actin polymerization. Analysis of cytosolic α-catenin by non-denaturing PAG showed dramatic decreases in free monomer and homodimer in both cases confirming that α-catenin was sequestered from the cytosol. These data confirm that the dynamic relationship between membrane-bound and cytosolic pools of α-catenin plays an important role in regulating actin dynamics and membrane activity. In addition, α-catenin appears to be a general regulator of cell dynamics at the single cell level, in addition to its role in cell-cell adhesion in a multicellular context.

127/B69

Dissection of α-catenin Organization and Function in Cells: I. Identification of α-catenin Monomer, Homodimer and αβ-heterodimers and Their Relationship with the Actin Cytoskeleton.

J. Nelson, J. Benjamin; Biology and MCP, Stanford University, Stanford, CA

Cadherins are cell-cell adhesion proteins that bind to cytoplasmic adaptor proteins (α- and β-catenin) and locally regulate the actin cytoskeleton. In vitro binding studies with purified proteins showed that α-catenin does not bind simultaneously to the cadherin/β-catenin complex and actin filaments. This mutually exclusive binding is explained by allosteric regulation of α-catenin in which monomer preferentially binds the cadherin/β-catenin complex, whereas α-catenin homodimer preferentially binds actin filaments and inhibits Arp2/3-mediated actin polymerization. The existence and potentially different functions of α-catenin monomers and dimers have not been examined in vivo. Based upon biochemical data, α-catenin in cells is predicted to reside in four functionally distinct pools: (1) cadherin-bound monomer; (2) cytoplasmic monomer; (3) cytoplasmic homodimer; and (4) cytoplasmic homodimer associated with actin. We fractionated MDCK cells into membrane and cytosol, and determined the distribution, binding partners and oligomer state of α-catenin. Alpha-catenin can be co-immunoprecipitated in a complex with E-cadherin and β-catenin from membranes in a ratio indicating that α-catenin exists as a monomer. Analysis of MDCK cell cytosol by non-denaturing PAG revealed 3 α-catenin species: the fastest migrating protein was in a complex with β-catenin (αβ-heterodimer); the two slower migrating proteins co-migrated with purified α-catenin monomer and homodimer, respectively. The migration of several putative α-catenin binding partners (actin, vinculin, Rho) were different from these α-catenin species. Significantly, relative amounts of α-catenin monomer and homodimer in the cytosol changed depending upon the state of actin polymerization in cells; actin depolymerization (with cytochalasin B) increased the amount of homodimer, presumably as a result of release from the actin cytoskeleton, while stabilization of actin (with jasplakinolide) reduced the amount of homodimer, presumably as a result of increased binding to the stabilized actin cytoskeleton. These results confirm in vitro studies that α-catenin exists as a monomer, homodimer, and a heterodimer with β-catenin, and that these species are functionally distinct.

128/B70

Neural Alpha-catenin Function in Cell-Cell Adhesion.

A. V. Kwiatkowski[1], C. C. Garner[2], W. Nelson[1]; [1]Biology, Stanford University, Stanford, CA, [2]Psychiatry and Behavioral Science, Stanford University, Stanford, CA

Classical cadherins are junctional proteins associated with the actin cytoskeleton and key regulators of cell-cell adhesion. The major intracellular components of cadherin junctions are the catenin proteins, with binding and recruitment of catenins to cadherins promoting cadherin-mediated adhesion. Association with the actin cytoskeleton is crucial for cadherin-mediated cell-cell adhesion, and the primary effector of cadherin signaling to the actin cytoskeleton is α-catenin, which binds directly to F-actin. The neuronal form of α-catenin, α-N-catenin, localizes to neuronal synapses - specialized cell-cell junctions that mediate intercellular signal transmission from one neuron to another - and regulates actin-dependent synapse morphology. However, the mechanism of how α-N-catenin acts downstream of cadherin engagement remains unknown. Here we describe a series of experiments designed to characterize the properties of α-N-catenin and investigate its role in cadherin-mediated cell-cell adhesion. We find both α-E-catenin - the epithelial version of α-catenin - and α-N-catenin expressed in cultured primary hippocampal neurons, and immunostaining and expression of fluorescently-tagged proteins reveal similar localization patterns. Both α-catenin proteins are abundant in brain lysate, though comparison of membrane and cytosol fractions reveal α-N-catenin solely in the membrane fraction whereas α-E-catenin is distributed between the cytosol and membrane fractions. FRAP analysis of MDCK cells stably expressing either EGFP- α-E-catenin or EGFP-α-N-catenin reveal a significant increase in the immobile fraction of α-N-catenin compared to α-E-catenin. Functional complementation tests conducted in MDCK cells by silencing endogenous α-E-catenin expression by RNAi and restoring α-catenin expression using rat α-E or α-N-catenin expose shared and unique abilities of α-E-catenin and α-N-catenin to regulate cell-cell adhesion. Together, these experiments reveal specific properties of α-N-catenin and offer further insight into the role of α-catenin in cadherin-mediated adhesion.

129/B71

A Novel Role for p120-Catenin in Anchorage-independent Cell Growth.

M. Dohn, M. V. Brown, A. Reynolds; Department of Cancer Biology, Vanderbilt University, Nashville, TN

p120-catenin (hereafter p120) regulates E-cadherin stability and has been suggested to function as a tumor suppressor. Here, we used anchorage-independent cell growth (AIG), a classic in vitro tumorigenicity assay, to examine the role of p120 in tumor initiation by several well-characterized oncogenes. Surprisingly, the AIG-inducing activities of both Rac1 and Src were completely blocked by removal of p120 by shRNA. This novel role for p120 was traced to its activity in suppression of the RhoA/ROCK pathway, which appears to be essential for the tumorigenic effects of both Rac1 and Src. Remarkably, the AIG block associated with p120 ablation was completely reversed by pharmaceutical inhibition of the downstream RhoA effector ROCK. H-Ras-induced AIG was also dependent on suppression of the ROCK/LIMK cascade but was p120-independent because its action on the pathway occurred downstream of p120. We conclude that p120 is an integral player in a pathway that directly controls AIG. Although the H-Ras pathway does not require p120, a unifying theme for all three oncogenes is that their pathways converge downstream to constitutively activate cofilin, which appears to be essential in MDCK and perhaps other cell types for acquisition of anchorage independence.

130/B72

Phosphorylation of Caveolin-1 Regulates Oxidant-induced Endothelial Hyperpermeability via Paracellular and Transcellular Pathways.

Y. Sun[1], R. Minshall[1,2], G. Hu[1]; [1]Pharmacology, University of Illinois College of Medicine, Chicago, IL, [2]Anesthesiology, University of Illinois College of Medicine, Chicago, IL

Caveolin-1, the primary structural and scaffolding protein of caveolae, is a key regulator of transendothelial permeability. Oxidants are known to increase endothelial permeability by opening interendothelial junctions, however, the mechanism is poorly understood. In this study, we tested whether H_2O_2-induced phosphorylation of caveolin-1 stimulates caveolae-mediated transcellular albumin transport and also regulates endothelial paracellular permeability. In rat lung microvascular endothelial cell line, H_2O_2 (100 μM ~ 5 mM) induced a concentration-dependent increase in caveolae-mediated endocytosis and transendothelial transport of ^{125}I-albumin which was dependent on the phosphorylation of caveolin-1. H_2O_2 induced stimulation of albumin permeability was blocked by either knockdown of caveolin-1 by specific siRNA or expression of non-phosphorylatable caveolin-1 mutant (Y14F-Cav-1). At the highest concentration tested (5 mM), H_2O_2 also induced the disassociation of E-cadherin/β-catenin complexes and decreased transendothelial monolayer electrical resistance, and this too was dependent on caveolin-1 phosphorylation. In the isolated wild type mouse lung, vascular perfusion of H_2O_2 induced caveolin-1 phosphorylation and lung edema formation, which was abolished in caveolin-1 null (*Cav-1*$^{-/-}$) lungs. Rescue of caveolin-1 expression in *Cav-1*$^{-/-}$ lung microvasculature by venous Cav-1 cDNA-liposome injection resulted in H_2O_2-induced lung edema formation, whereas expression of Y14F-Cav-1 did not. Thus, oxidant-induced phosphorylation of caveolin-1 mediates H_2O_2-induced vascular albumin and fluid hyperpermeability by stimulating caveolae-mediated transcytosis and destabilizing adherens junctions, respectively. Furthermore, these data suggest that tyrosine phosphorylation of caveolin-1 may be a therapeutic target in the treatment of oxidant-induced lung microvascular injury and protein-rich pulmonary edema. (Supported by NIH HL71626 and HL60678, and AHA 0730331N).

131/B73

Characteristics of the Signal Peptide of Adherence Junction Protein Shrew-1 and Potentials for Novel Regulatory Mechanisms.

A. Schreiner[1], E. Resch[1], J. A. Hiss[2], S. Quaiser[3], T. Quaiser[4], J. Gross[1], M. Meissner[2], G. Schneider[2], A. Starzinski-Powitz[1]; [1]Department of Molecular Cell Biology and Human Genetics, Goethe University, Frankfurt am Main, Germany, [2]Department of Chem- and Bioinformatics, Goethe University, Frankfurt am Main, Germany, [3]Department of Biochemistry, RWTH Aachen University, Aachen, Germany, [4]Process Systems Engineering, RWTH Aachen University, Aachen, Germany

Shrew-1 is a vertebrate specific membrane protein involved in the modulation of cellular invasion and a constituent of adherence junctions in polarized epithelial cells. It consists of 411 aminoacids (aa) with a rather peculiar primary structure and has no homology with known

proteins. Both, the unusually long signal peptide (SP; 43 aa) of shrew-1 and its transmembrane segment (TMS) were shown to exhibit anticipated activities using secreted alkaline phosphatase (SEAP) as a reporter protein. The predicted SP was able to functionally replace that of SEAP and the TMS acted as stop transfer signal. However, the expected plasma membrane localisation of reporter proteins containing the TMS was only observed if the TMS was paired with shrew-1's SP while SEAP's SP was not able to achieve it. This possibly reflects direct or indirect interactions between shrew-1's SP and TMS at some level. More detailed computational analyses of shrew-1's SP revealed a novel SP domain organization giving rise to the NtraC model. In this, the SP is subdivided into an N-terminal and C-terminal domain which both are interconnected by a turn-rich transition area (tra). It was predicted that the C-domain accommodates the activity for translocation into the endoplasmic reticulum, whereas the N-domain alone should be able to act as a mitochondrial transit peptide. Using the appropriate SEAP fusion proteins, the C-domain of shrew-1 could indeed replace SEAP's SP leading to secretion of the protein, and the N-domain led to translocation of some SEAP protein into mitochondria as predicted by the computational analysis. Finally, searching the UniProtKB database for proteins that contain N-terminal signal peptides with more then 40aa led to the identification of 22 single pass transmembrane proteins with an NtraC organization analogous to that of shrew-1. This implies that shrew-1 belongs to a novel and general type of protein which contains multiplex SP being able to exhibit different targeting and/or regulatory functions.

132/B74

A Novel Type of Adhering Junction in Transformed Mesenchymal Cells: Plakophilin-2 in a Non-epithelial Context.

S. Rickelt, W. W. Franke; Helmholtz Professor Group for Cell Biology, German Cancer Research Center, Heidelberg, Germany

The plaque-bearing adhering junctions (AJs) of epithelial cells are commonly divided into the intermediate filament-associated desmosomes (maculae adhaerentes) and the microfilament-anchoring AJs, including the puncta adhaerentia. The molecular composition of both kinds of AJs is by and large known and the specific antibodies are generally used in cell biology and pathology. By comparison, little is known as to the molecular composition of the AJs that connect the cells of mesenchymally derived tissues and tumors. In our studies of normal and SV40-transformed mesenchymally derived cells and soft tissue tumors we have identified a novel cell type-specific AJ ("iunctura intermedia"). Using biochemical, immunological as well as light and electron microscopical techniques we show that AJs of both the normal and the transformed human cells are characterized by the transmembrane glycoprotein N-cadherin, in some cells together with cadherin-11 and nectin, in combination with the plaque proteins α- and β-catenin, p120 and plakoglobin. Surprisingly, however, we noted in malignantly transformed cells the systematic addition of plakophilin-2, a plaque protein normally found in desmosomes and the composite junctions of cardiomyocytes, which is known for its junction-organizing role. Interestingly, this new cell-cell junction type was also found in a diversity of malignantly transformed cell culture lines as well as some soft tissue tumors in situ. Further research will be necessary to elucidate the mechanisms by which plakophilin-2 influences the functions of the AJ ensemble, and first results of function-oriented experiments will be presented.

133/B75

Plakophilin 2 Integrates Intracellular Signals Required for the Coordinated Remodeling of the Intermediate Filament and Actomyosin Cytoskeletons Necessary for Desmosome Assembly.

L. M. Godsel, A. E. Bass-Zubek, R. P. Hobbs, N. J. Garcia, K. J. Green; Pathology, Northwestern University, Chicago, IL

Members of the plakophilin (PKP) subfamily of armadillo proteins are components of desmosomes, intercellular junctions essential for maintaining the integrity of epithelia and heart. PKP2 associates with the intermediate filament (IF) anchoring protein desmoplakin (DP) in cytoplasmic precursors during junction assembly. We previously showed that PKP2 knock down (KD) interferes with IF network reorganization and DP precursor translocation to junctions. Aberrant precursor dynamics were due at least in part to stabilization of normally dynamic DP -IF interactions, which depend on regulation by PKP2-scaffolded PKCα. Since cytochalasin D also blocks precursor translocation, we investigated whether PKP2 additionally plays a role in actin remodeling events required for desmosome assembly. Our data show that both pharmacological inhibition of myosin II and PKP2 KD interfere with DP incorporation into junctions without affecting E-cadherin accumulation at cell-cell borders. Loss of PKP2 altered multiple phases in the temporal program of actomyosin rearrangements triggered by cell-cell contact. At the onset of junction assembly in control cells, RhoA was recruited to cell-cell contacts within 30 minutes. Recruitment was inhibited in PKP2 KD cells, suggesting a loss of membrane-proximal contractile signaling that could contribute to impaired DP precursor translocation. During junction maturation and at steady state, however, biochemical indicators of contractile signaling were elevated, as reflected by increased RhoA and myosin activity (Ser19 myosin light chain phosphorylation) and myosin phosphatase inactivation (Thr696 phosphorylation). These alterations were accompanied by retention of DP particles and IF within a circumferential actin ring that failed to reorganize peripherally into a normal cortical distribution and maintenance of radially oriented actin stress fibers. The data support a model whereby PKP2 functions as a scaffold to integrate PKCα and RhoA signaling for coordinating the dynamic cytoskeletal rearrangements necessary to drive IF reorganization and desmosome assembly.

134/B76

p120 Ablation Dramatically Increases Tumor Incidence in APC Mutant Mice.

W. Smalley[1], P. Burnett[2], M. A. Davis[1], M. Washington[3], S. Robine[4], D. L. Gumucio[5], R. Coffey[6], A. B. Reynolds[1]; [1]Cancer Biology, Vanderbilt University, Nashville, TN, [2]Dermatology, Vanderbilt University, Nashville, TN, [3]Pathology, Vanderbilt University, Nashville, TN, [4]Pathology, Institut Curie, Paris, France, [5]Cell and Developmental Biology, University of Michigan, Ann Arbor, MI, [6]Medicine, Vanderbilt University, Nashville, TN

Pathology studies report regions of p120 loss in 5 - 20% of human colorectal tumors. Here, we have used conditional p120 knockout mice and Cre-lox technology to target p120 ablation to intestinal epithelia in normal mice and in littermates expressing mutant APC alleles. Interestingly, constitutively active p120 ablation (Villin-Cre; p120F/F) induced adhesive defects and bleeding that was lethal by 17 days of age. To circumvent early lethality, we turned to a tamoxifen-inducible Cre model (Vil-Cre-ERT2; p120F/F), and established conditions that limited p120 loss to 10% of the intestinal epithelia. These mice were examined at regular intervals up to 18 months of age. As before, E-cadherin was significantly downregulated in p120-null cells, and cell morphology and adhesion was perturbed. By two months, however, all

knockout mice also contained numerous cystic crypts in both the small intestine and colon. By 12-18 months, 30% of mutant mice developed adenomas in the small intestine, cecum, and/or colon. Thus, limited p120-ablation by itself induces tumors in approximately 1/3 of mutant animals. To examine p120 ablation in the context of APC mutation, we introduced a mutant APC allele (APC1638N) to obtain Vil-Cre-ERT2; p120F/F; APC1638N mice. At nine months, APC1638N mice averaged 5 adenomas per animal, whereas littermates with limited p120 ablation averaged 50 adenomas per animal. Thus, even limited p120 ablation increases the potency of the APC1638N allele by more than ten-fold.

135/B77

Uncovering the Mechanisms of Epidermal Differentiation vs. Proliferation.

S. H. Lasse, C. Jamora; Biological Sciences, UCSD, La Jolla, CA

The maintenance and repair of many tissues and organs relies on a specified pool of stem cells that have the ability to proliferate and differentiate. The balance between these two fates is critical for maintaining homeostatic numbers of stem cells and tissue cells. Using the epidermis as a model we set out to examine the molecular mechanisms behind the balance of proliferation and differentiation. Previous work by the Gumbiner laboratory showed that E-cadherin can inhibit cell proliferation. We overexpressed E-cadherin in the epidermis of mice and observed early entry of epidermal stem cells into the terminal differentiation program, suggesting that E-cadherin also regulates differentiation. This finding supports previous conditional deletions of E-cadherin in the epidermis by the Fuchs and Suter laboratories. Loss of E-cadherin in the epidermis results in hyperproliferation and failure to fully execute the terminal differentiation program. Reconstitution of this process in vitro with cultured epidermal stem cells allows the dissection of how E-cadherin influences differentiation. Overexpression of different domains of E-cadherin reveals that the induction of differentiation requires the beta-catenin binding capacity of E-cadherin and is independent of cell-cell adhesion. With this information we can probe further into the mechanism through which adhesion at the plasma membrane can influence cell fate.

136/B78

The Adhering Junctions of Valvular Interstitial Cells.

M. Barth, W. W. Franke; Helmholtz Professor Group for Cell Biology, German Cancer Research Center, Heidelberg, Germany

Great efforts have been made in the growing field of heart valve tissue engineering to improve the outcome of patients who underwent heart valve transplantation. Thus it is surprising to see how limited our cell and molecular biological knowledge about valves and the valvular interstitial cells (VICs) still is, notably with respect to their cell-cell contacts. Therefore, we have determined in different mammalian species, the molecules contributing to the adhesion between VICs in situ and in cell culture. Using biochemical and immuno-localization methods we have found that the adhering junctions (AJs) between VICs are composed of proteins with characteristics similar to those of other non-epithelial AJs: In addition to the transmembrane glycoprotein N-cadherin and the typical plaque proteins α- and β-catenin, we have identified cadherin-11 as well as the plaque proteins plakoglobin and p120ctn in heart valves, as well as in cell cultures. Specifically, we have excluded the occurrence of desmogleins, desmocollins and desmoplakin. Surprisingly, in VICs of primary cultures but not in those of valve tissues, we have noted that their AJs contain the desmosomal plaque protein plakophilin-2. Moreover, the distribution of VICs in situ is strikingly different from that seen in monolayer cultures: While most of the VICs in situ are dispersed, with remarkable intercellular distances, cultured VICs grow to monolayers of densely packed cells connected by frequent AJs of the composition described, suggesting the existence of molecules acting as "VIC scatter factors" in the tissue, which has not been identified yet. Furthermore, VICs in situ form frequent, often long filopodial cell processes and are accompanied by paracrystalline collagen bundles in the extracellular matrix. By electron microscopy, we have found a systemic association of the VIC filopodia with these collagen bundles, often in semi-ordered patterns. This regular association of VICs with collagen bundles resembles the collagen fiber associations of fibroblastoidal cells in tendons as well as in diverse other kinds of tissues. We introduce these specific filopodial collagen attachment structures as a novel and widespread cell-matrix organization element.

137/B79

Early Steps in Adherens Junction Assembly in the Drosophila Embryo: Investigating Stepwise Roles for Microvilli and Bazooka/Par-3.

M. A. McGill, A. McKinley, T. Harris; Cell and Systems Biology, University of Toronto, Toronto, ON, Canada

Epithelial cells are linked together by multi-protein complexes called adherens junctions (AJs). AJs are composed of cadherin (DE-Cad) receptors linked to cytoplasmic β-catenin/Armadillo (Arm), α-catenin and actin and are critical for epithelia structure and function. As the first epithelium forms in *Drosophila*, the polarity cue Bazooka (Baz) localizes to the apical domain where it somehow positions precursory AJs (spot junctions, SJs). We sought to characterize the dynamics of and define the molecular mechanisms by which Baz regulates AJs assembly. Much of our understanding of this assembly is inferred from immunofluorescence studies in fixed tissues that provide only a static representation in which some structures may not be detected. Using live cell imaging of *Drosophila* embryos expressing DE-CadGFP, ArmGFP or BazGFP, we identified novel DE-Cad, Baz and Arm puncta over the apical cell surface. These complexes localize between actin microvilli and are present from early to mid cellularization but decrease in number as SJs form between lateral membranes by gastrulation. Without Baz, these apical complexes formed normally but abnormally persist into gastrulation. This suggests a two step assembly pathway whereby an actin based process forms apical DE-Cad and Arm complexes in early SJ assembly and Baz clusters these preformed complexes at lateral sites of SJ assembly. To further delineate Baz's role in SJ assembly we characterized the molecular dynamics of DE-Cad, Arm and Baz within SJs using fluorescence recovery after photobleaching and combined this with absolute counts of each molecule in SJs to determine the number of molecules entering a SJ per second. Compared to Baz, a greater number of DE-Cad and Arm molecules enter a SJ per second. This and other data argues against single molecules of Baz recruiting single DE-Cad and Arm molecules into SJs, but is consistent with a role for Baz in clustering preformed clusters of DE-Cad and Arm.

138/B80

Counting DE-Cadherin, Armadillo, and Bazooka/PAR-3 Molecules in a Spot Junction during Drosophila Embryogenesis.

A. McKinley, M. A. McGill, T. Harris; Cell and Systems Biology, University of Toronto, Toronto, ON, Canada

Epithelial tissue provides a critical barrier between internal compartments and the surrounding environment, creating distinct anatomical compartments. For epithelia to form properly, cells must adhere to one another in a polarized fashion, via adherens junctions (AJs), consisting of transmembrane cadherins (DE-Cadherin, (DE-Cad)) and cytoplasmic catenins (β-catenin (Arm) and α-catenin). In *Drosophila*, the scaffold protein Bazooka (Baz) localizes apically and is responsible for positioning spot junctions (SJs, early AJs) to the apical domain, but the mechanism is unclear. To understand how any machinery works it is important to know the numbers of its component parts. Thus, we sought to count the number of DE-Cad, Arm, and Baz molecules within a SJ. First we used live microscopy to measure the relative fluorescent intensities of GFP fusions of each protein within SJs and the intervening membrane at late cellularization. Second, using ELISA with a GST-GFP standard curve, we determined the number of molecules/embryo for each of three GFP proteins. Knowing the number of cells/embryo and the number of SJs/cell, we calculated the number of molecules of each protein within a SJ. This indicated Baz forms a 1:7 ratio with DE-Cad and Arm, a ratio confirmed with direct relative fluorescent intensity measurements. To determine whether Baz might interact with clusters of DE-Cad and Arm formed prior to SJ assembly, we re-examined the localization of each protein using live microscopy. We found early complexes of DE-Cad, Arm, and Baz over the apical surface, not previously seen in fixed images. During SJ assembly, the number of apical surface complexes decreases as lateral SJs increase. Without Baz, DE-Cad is mislocalized apically and basally and forms smaller preformed complexes. We hypothesize that Baz may cluster preformed complexes of DE-Cad and Arm as part of their re-positioning from the apical surface to sites of lateral junction assembly.

139/B81

Investigation of the p120-catenin/E-cadherin Interaction and the Stability of E-cadherin in MDCK Cells.

A. E. Hartsock[1], W. Nelson[1,2]; [1]Molecular and Cellular Physiology, Stanford University, Stanford, CA, [2]Biological Sciences, Stanford University, Stanford, CA

E-cadherin, a component of the Adherens Junction, initiates the formation of cell-cell contacts. The stability of E-cadherin is necessary to maintain adhesive contacts during tissue development, but during tissue reorganization adhesive contacts and are modified to allow increased cell dynamics. p120 catenin binds to the juxtamembrane domain (JMD) of E-cadherin, and this interaction has been proposed to stabilize E-cadherin, whereas uncoupling of this interaction induces endocytosis of E-cadherin. Upon endocytosis, E-cadherin may be recycled back to the membrane or degraded. The mechanism by which E-cadherin is degraded is unknown. To examine how p120 regulates E-cadherin stability and the mechanism of E-cadherin degradation, the JMD of murine E-cadherin (94 amino acids), tagged with monomeric Red Fluorescent Protein (RFP) was targeted to the mitochondria through an ActA peptide sequence (JMD-ActA) in MDCK cells. In these cells, JMD-ActA localizes with mitochondrial markers in both fixed and live cells, and levels and location of endogenous proteins at the plasma membrane were unaffected. p120 did not accumulate at the mitochondria. Decreased amounts of JMD-ActA at the mitochondria were observed in comparison to a control vector, indicating that JMD-ActA might be unstable and degraded. To assay for degradation, MDCK cells expressing either JMD-mRFP-ActA or mRFP-ActA were incubated 6 hours with 10μM MG-132. Western Blot analysis of whole cell lysates demonstrated an increase in protein levels of JMD-ActA upon MG-132 treatment, but not mRFP-ActA. p120 did not localize at mitochondria in JMD-ActA stably expressing cells even after MG-132 incubation. However transient expression of the JMD at the plasma membrane presents increased levels of p120. In addition, mutation of the lysine to argenine residues within the JMD stabilized the JMD at the mitochondria and lead to the recruitment of p120. The data suggest that the JMD of E-cadherin is subject to degradation via the proteasome when p120 is not bound in a ubiquitin dependent manner. It remains to be determined if the stability of the JMD allows for the recruitment of p120 or the binding of p120 promotes the stability of the JMD.

141/B83

Mutation of Lyst in Mice Recapitulates Aspects of Human Exfoliation Syndrome.

C. M. Trantow[1], M. Anderson[1,2]; [1]Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA, [2]Ophthalmology and Visual Sciences, University of Iowa, Iowa City, IA

Exfoliation syndrome (XFS) is a common age-related disorder primarily recognized by the pathological accumulations of a fibrillar exfoliative material in the anterior chamber of the eye. Human eyes with XFS exhibit a striking pattern of Marcel-like iris transillumination defects. The same pattern is recapitulated in mice containing the *Lyst(bg-J)* mutation. The goal of these experiments was to capitalize on this resource by using mice to begin studying the molecular pathways of XFS. B6-*Lyst(bg-J)* mice were identified from a screen of coat color variants assayed by slit-lamp exam. B6-*Lyst(bg-J)* mice and age-matched C57BL/6J control mice were subsequently analyzed by clinical, histological, and physiological assays. The iris transillumination defect in both human XFS patients and B6-*Lyst(bg-J)* mice correlate to an unusual "saw-tooth" morphology of the iris pigment epithelium. Similar to human XFS, B6-*Lyst(bg-J)* mice produce an exfoliative-like material and exhibit pronounced pigment dispersion. B6-*Lyst(bg-J)* mice also produce accumulations of PAS-positive and anti-fibrillin-1 positive material, which contribute to occlusion of the irideocorneal angle. We have found the molecular basis of the *bg-J* mutation is a result of a three base pair deletion in the WD40 encoding region of the *Lyst* gene. Previously it has been shown that the LYST WD40 domain interacts with CSNK2B. We confirmed this interaction with a GST-pulldown experiment. Interestingly, LYST(bg-J) completely disrupts the interaction with CSNK2B. These results lead to a working hypothesis that aspects of the XFS phenotype involve LYST and CSNK2B pathways, likely influencing cell-cell adherens junctions. B6-*Lyst(bg-J)* mice represent the first animal model that recapitulates any aspect of XFS. Our results suggest that LYST, or a component of the LYST genetic pathway, are excellent candidates for contributing to human XFS and represent a valuable resource for studying the disease pathway.

142/B84

Functional Analysis of the PET-LIM Protein LMO6 and Implications for Cellular Adhesion.

S. Hernandez[1], E. Jakobsson[1], I. Martin-Ruiz[1], L. de Iñigo Mojado[1], F. Elortza[2], J. Sutherland[1]; [1]Lab 2, Proteomics Unit, CIC-bioGUNE, Derio, Spain, [2]Proteomics Service, CIC-bioGUNE, Derio, Spain

PET-LIM proteins such as TES and Prickle have roles in cell polarity, adhesion and tumor suppression. Previous work suggests that PET-LIMs may serve as regulated adaptors for organizing protein complexes using intramolecular conformational changes and scaffold assembly on the multiple LIM domains. We present our analysis of LMO6, a novel member of the PET-LIM family. In addition to the PET-LIM domains, LMO6 combines sequence features seen in either TES or Prickle proteins, suggested that it has a novel function, further supported by its presence only in vertebrates. LMO6 has a multidomain architecture (PET domain, three LIMs and an extended C-terminal domain, or TAIL), and fusions with GFP give rise to distinct localisations within the cell (e.g. cell adhesions, subnuclear bodies, nuclear envelope). The TAIL domain contains a functional bipartite NLS, which may be regulated by serine phosphorylation, and a farnesylation motif, which is needed for membrane localisation. We have identified candidate interactors of LMO6 using TAP-tagging/mass-spectrometry and these partners suggest a role for LMO6 complexes in cell adhesion, RNA transport and mitosis. Like other LIM proteins, LMO6 also shuttles between cytoplasm and nucleus, perhaps mediating cell signaling. Moderate expression levels of LMO6 mRNA were observed by qRT-PCR in a panel of human tissues, but higher levels were observed in skin. Thus, LMO6 protein localisation was analysed in primary keratinocytes. Undifferentiated cells (low Ca++) show a diffuse cytoplasmic signal. However upon differentiation (high Ca++), there is a transient accumulation at the nuclear envelope and "adhesion zipper" filopodia (6 hours), and eventually is deposited at cell-cell junctions in mature adhesions (24 hours). Similar localization of LMO6 to cell-cell junctions and nuclear envelope is seen in other epithelial cell lines (HaCat keratinocytes, HeLa, T47D breast carcinoma). Overexpression of GFP-LMO6 in T47D cells reduced their ability to form colonies in soft-agar, however does not affect their growth properties (in contrast to GFP-TES). Taken together, our results suggest that LMO6 may regulate cell adhesion assembly/stability.

Focal Adhesions (143-161)

143/B85

The Attachment of Cells to Surfaces Exclusively via Syndecan-4 Induces the Recruitment of Both Vinculin and Talin.

E. Morse, M. Frigault, M. McKenna, R. Bellin; Dept. of Biology, Holy Cross College, Worcester, MA

Syndecans are a class of transmembrane heparan sulfate proteoglycans that have been long known to be involved in cellular adhesion. In particular, syndecan-4 has been shown to exist at sites of focal adhesions, normally in addition members of the integrin family of transmembrane proteins. In our continuing studies, we have explored the precise role of syndecan-4 in cell adhesion by developing various surfaces coated with immobilized anti-syndecan-4 antibodies in order to promote syndecan-specific adhesion independent of integrin binding. In previous work, we demonstrated that syndecan-4 antibody-mediated cellular attachment to surfaces acts to recruit the focal adhesion protein vinculin to adhesion sites without requiring the attachment of β1 integrin molecules to the antibody coated surface. Confocal microscopic analysis confirmed that cells bound to surfaces coated with syndecan-4 antibody were able to promote the development of vinculin-containing contacts that lack the presence of β1 integrins, but still allow normal cell spreading. In our current studies we have extended this work by using anti-syndecan-4 coated microbeads and coverslips to demonstrate that the focal adhesion protein talin is also recruited to sites of syndecan-4-specific cell adhesion. These data indicate that binding of the core protein of syndecan-4 is sufficient to initiate the formation of focal adhesions and promote cell spreading independent of β1 integrin binding.

144/B86

Functional Analysis of the TES Tumor Suppressor Reveals Important Determinants for Molecular Interactions and Nucleocytoplasmic Shuttling.

E. Jakobsson, I. Martin-Ruiz, A. Mohry, J. Sutherland; Lab 2, Proteomics Unit, CIC-bioGUNE, Derio, Spain

TES (or Testin) was first described as a locus that undergoes frequently LOH in human cancers, and therefore was touted as a tumor suppressor. Indeed, a KO mouse model of TES confirms that this protein has tumor suppressor activity. TES is a focal adhesion protein with roles in cell spreading and attachment to extracellular matrix and belongs to a family of PET-LIM proteins. LIM domains have been well characterized due to their frequent use as protein interaction modules. The PET domain, however, is conserved and uniquely found in association with LIMs, suggesting that the two domains may have an inseparable relation, most likely via intramolecular contacts. Using TES as a model, we are taking several approaches to better understand the structure/function of the PET domain and its relation with LIMs and other motifs. TES plays its role by interaction with other proteins (zyxin, paxillin, Mena) as being part of a multiprotein complex at the focal adhesions, possibly as a scaffold or blocking access for other partners. Based on previous results we suspect that TES is a dynamic protein that might regulate its functions by conformational changes. Using GFP fusions, we have observed that point mutations in the PET domain can affect the localization in HeLa cells. These mutants strongly localize to the focal adhesions whereas wild-type TES is diffuse throughout the cytoplasm, with variable amounts seen in the nucleus and on the stress fibres, with enrichment at the focal adhesions. We propose that these mutations disrupt the intramolecular interactions between the PET and LIM domains and result in an "open conformation", which might be the required conformation for TES to go to the focal adhesions. As other LIM proteins, we have shown that TES is shuttling between the nucleus and cytoplasm, suggesting a role in adhesion-based signaling. We have identified a signal that regulates the nuclear localization of TES, as well as a conserved Cys-rich domain that may mediate the binding/transport of TES on actin microfilaments. Our findings will contribute to unraveling the molecular mechanism behind the tumor suppressor properties of TES.

145/B87

dTES, a Drosophila Homolog of the Tumor Suppressor Testin, Is Required for Gametogenesis.

J. Sutherland, J. Blanco-Calvo, I. Martin-Ruiz, L. de Iñigo Mojado; Lab 2, Proteomics Unit, CIC-bioGUNE, Derio, Spain

We present our analysis of *Drosophila* TES (dTES), a fly homolog of the focal adhesion protein TES (or Testin), which has tumor suppressor properties in vertebrates. TES is thought to be a flexible adaptor protein that contributes to focal adhesion assembly/disassembly. While

dTES is expressed throughout development, it is enriched in both somatic and germ cells of the developing testes and ovaries. Antibody staining reveals dTES at cellular adhesions as well as putative "organelle-attachment sites" within cells. We have identified a mutation in dTES, consisting of a P-element insertion (EP) in the promoter region, which results in pleiotropic effects, including embryonic lethality and few adult escapers. The homozygous adults have defects in gametogenesis, with complete sterility in males and reduced fertility in females. Mutant males show a striking defect in spermatogenesis characterized by a loss of coordination of the actin-based individualization complex. Egg chambers show a range of phenotypes, including ring canal detachment and weakening of the follicular epithelium. These phenotypes are consistent with the observed dTES localization in these tissues. In mice, loss of TES does not impair development or fertility, suggesting either it is unnecessary, or it has redundant function to other TES-like proteins. However, TES function is revealed in carcinogenesis; loss of a single copy of TES predisposes mice to aggressive gastric cancers in a similar manner than loss of both copies, suggesting that tumor suppression by TES is haplosensitive. In flies, our rescue experiments using dTES transgenes suggest that normal dTES function may also be sensitive to gene dosage, since extra copies of dTES lead to distinct earlier defects in spermatogenesis. For decades, no chromosomal deficiencies were available for the region where dTES is located, indicating that a haploinsufficent locus may reside there. However, we are characterizing recently isolated genetic deficiencies (from K. Cook, Bloomington) to see whether loss of a single copy of dTES can affect gametogenesis. Our study expands the functions attributable to PET-LIM proteins and will allow detailed functional analysis of dTES domains *in vivo*.

146/B88

Stability of Adhesion Cluster and Cell Reorientation under Lateral Cyclic Tension.

D. Kong[2], B. Ji[1], L. Dai[2]; [1]Institute of Biomechanics and Medical Engineering, Department of Engineering Mechanics, Tsinghua University, Beijing, China, [2]State Key Laboratory of Nonlinear Mechanics, Institute of Mechanics, Chinese Academy of Sciences, Beijing, China

This work is motivated by the experimental observations of different responses of cell on stretched substrate to the static and dynamic loads. A model of focal adhesion which can consider the mechanics of stress fiber, adhesion bonds, and substrate is developed at molecular level by treating the focal adhesion as an adhesion cluster. The stability of the cluster under dynamic load is studied by applying cyclic external strain on the substrate. We show that there exists a threshold value of external strain amplitude, beyond which the adhesion cluster disrupts quickly. In addition, our results show that the adhesion cluster is prone to losing stability under high-frequency loading, because the receptors and ligands can not get enough contact time to form bonds due to the high-speed deformation of the substrate, and at the same time the viscoelastic stress fiber becomes rigid at high-frequency which attributes large deformation to the bonds. Furthermore, we find that the stiffness and relaxation time of stress fiber take important roles in the stability of the adhesion cluster. The essence of this work is to connect the dynamics of the adhesion bonds (molecular level) with the behaviors of the reorientation of cell (cell level) through mechanics of stress fiber. The predictions of the cluster model are consistent with the experimental observations.

147/B89

Actin Polymerization at Focal Adhesions Is Facilitated by Mechanical Forces in a Zyxin-dependent Manner.

H. Hirata[1,3], H. Tatsumi[2], M. Sokabe[1,2,3]; [1]Molecular Physiology, National Institute for Physiological Sciences, Okazaki, Japan, [2]Physiology, Nagoya University Graduate School of Medicine, Nagoya, Japan, [3]Cell Mechanosensing Project, Japan Science and Technology Agency, Nagoya, Japan

The transmembrane adhesive molecules integrins are clustered at focal adhesions (FAs) forming tight bindings to extracellular matrices, while they link to actin cytoskeleton at their cytoplasmic domains. FAs serve as sites for actin polymerization, and accumulated F-actin presumably strengthens the integrin-actin cytoskeleton linkage. On the other hand, the linkage between integrin and actin cytoskeleton is regulated by intra- and extracellular mechanical environments. Therefore, it is conceivable that actin polymerization at FAs is affected by mechanical forces, however, their exact role on actin polymerization remains unknown. To address this issue, we assessed actin polymerization at FAs by introducing fluorescence-labeled actin molecules into digitonin-permeabilized human skin fibroblasts cultured on fibronectin. When cell contractility was inhibited by the myosin II inhibitor blebbistatin, actin polymerization at FAs was diminished, while α5β1 integrin remained accumulated at FAs. To examine the action of mechanical forces more directly, blebbistatin-treated cells were subjected to a sustained uniaxial stretch, which induced the actin polymerization at FAs. These results demonstrate the novel role of mechanical forces in actin polymerization at FAs. To reveal the molecular mechanism underlying the force-induced actin polymerization at FAs, we examined the distribution of zyxin, a postulated actin-regulatory protein. Actin polymerizing activity was strong at FAs with large amounts of zyxin. Accumulation of zyxin at FAs was diminished by blebbistatin, while uniaxial stretching of the cells induced the zyxin accumulation. When endogenous zyxin was displaced from FAs by overexpressing the C-terminal LIM region of zyxin that is responsible for targeting zyxin to FAs, the force-induced actin polymerization at FAs was decreased. These results demonstrare that zyxin is involved in the mechanical force-dependent facilitation of actin polymerization at FAs.

148/B90

The Role of PIP5 Kinases in the Regulation of the Actin Cytoskeleton.

A. Corgan[1], Y. Mao[1], X. Zhu[1], Y. Wang[1], M. Yamaga[1], K. Luby-Phelps[2], G. Di Paolo[3], P. De Camilli[4], H. Yin[1]; [1]Department of Physiology, UT Southwestern, Dallas, TX, [2]Department of Cell Biology, UT Southwestern, Dallas, TX, [3]Department of Pathology, Columbia University, New York, NY, [4]Howard Hughes Medical Institute and Department of Cell Biology, Yale University, New Haven, CT

Phosphatidylinositol-4,5-bisphosphate (PIP2), an important regulator of the actin cytoskeleton and plasma membrane functions, is primarily synthesized by the type I phosphatidylinositol 4 phosphate 5 kinases (PIP5Ks). Mammals have three PIP5K genes (PIP5Kα, PIP5Kβ, and PIP5Kγ), and the γ isoform has a 90 kDa and an 87 kDa splice variant. We found that the depletion of each PIP5K isoform individually by RNA interference (RNAi) or gene knockout (KO) by homologous recombination generated distinct changes in the actin cytoskeleton and signaling responses. The actin phenotype of the PIP5Kγ depletion (using pan siRNA, which is directed against a common sequence shared by the 90 and 87kDa isoforms) in HeLa cells is particularly striking: it results in increased actin stress fibers, decreased chemotaxis, and increased adhesion to fibronectin-coated substrates. There is also a striking increase in prominent focal adhesions (FA). Using real-time

interference reflection microscopy, we found that the turnover of FA is 48% slower in the PIP5Kγ depleted cells. Likewise, there is a large decrease in the dynamic turnover of GFP-labeled vinculin and paxillin in FA, as monitored by fluorescence recovery after photobleaching. Since PIP5Kγ90 has already been implicated in FA assembly, we depleted it specifically without depletion of the much more abundant PIP5Kγ87 by using a PIP5Kγ90-specific targeting sequence. This fails to produce robust stress fibers. In addition, PIP5Kγ87 overexpression per se is able to rescue the actin phenotype in HeLa cells with pan PIP5Kγ knockdown. Similar results were obtained in mouse embryonic fibroblasts (MEF) from PIP5Kγ KO mice. Thus, PIP5Kγ87 is the major contributor to the pan PIP5Kγ depletion/knockout robust actin and FA phenotype. We sought to identify the molecular mechanisms of the PIP5Kγ depleted actin phenotype. Inhibitors of myosin, ROCK, and Rho all decreased the amount of thick actin stress fibers, suggesting that the phenotype is due to abnormal Rho activation. Furthermore, Rho A activity is elevated in PIP5Kγ depleted cells. We hypothesize that PIP5Kγ regulates the actin cytoskeleton by inhibiting Rho, and thus its downstream effectors ROCK and myosin.

149/B91

Focal Adhesion Kinase Is Important for Fluid Shear Stress Induced Mechanotransduction in Osteoblasts.

S. Young, F. Pavalko; Physiology, IU School of Medicine, Indianapolis, IN

Mechanical loading of bone is important for maintenance of bone mass and structural stability of the skeleton. When bone is mechanically loaded, movement of fluid within the spaces surrounding bone cells generates fluid shear stress (FSS) that stimulates osteoblasts resulting in enhanced anabolic activity. The mechanisms by which osteoblasts convert the external stimulation of FSS into biochemical changes, a process known as mechanotransduction, remain poorly understood. Focal adhesions are prime candidates for transducing external stimuli. Focal adhesion kinase (FAK), a non-receptor tyrosine kinase found in focal adhesions, may play a key role in mechanotransduction, althought its function has not been directly examined in osteoblasts. We examined the role of FAK in osteoblast mechanotransduction using short interfering RNAs (siRNA), over-expression of a dominant negative FAK (FRNK) and FAK-/- osteoblasts to disrupt FAK function in calvarial osteoblasts. Osteoblasts were subjected to varying periods oscillatory fluid flow (OFF) from 5 minutes to 4 hours and several physiologically important readouts of mechanotransduction were analyzed including: ERK1/2 phosphorylation, upregulation of c-Fos, COX-2 and OPN, and release of PGE2. In this study we determined that osteoblasts with disrupted FAK signaling exhibited a 43-77% reduction of ERK1/2 phosphorylation after exposure to 5, 15 and 30 minutes of OFF as compared to control osteoblasts also exposed to OFF. In addition, c-Fos upregulation after 30 minutes of OFF was reduced 24-50%, and the upregulation of COX-2 in response to 4 hours of OFF was decreased 50-70% in osteoblasts with disrupted FAK signaling. The reduced COX-2 expression in FAK impaired osteoblasts resulted in a 34-47% decrease in PGE2 release. Finally, the expression of OPN, a bone remodeling protein, was reduced 35-80% following 2 hours of OFF in FAK impaired osteoblasts. In addition, we determined that Pyk2, a FAK family member, activation and expression were not affected by disruption of FAK signaling in osteoblasts. These data indicate the importance of FAK during FSS induced mechanotransduction in osteoblasts.

150/B92

Cdk5 Is Essential for Focal Adhesion Assembly in Spreading Corneal Epithelial Cells.

Q. Pan, C. Y. Gao, P. Zelenka; National Eye Institute, NIH, Bethesda, MD

Cdk5 is a proline-directed Ser/Thr kinase, which regulates adhesion and migration in a variety of cell types, including epithelial cells of the lens and cornea. Since signaling through integrin-dependent focal adhesions is central to both adhesion and migration, the present study examines the possible involvement of Cdk5 in focal adhesion assembly in spreading human corneal limbal epithelial (HCLE) cells. Immunofluorescence of spreading cells 60 min after plating on fibronectin showed that Cdk5(pY15) and the Cdk5 activating protein, p35, co-localized with FAK, Src(pY416), and vinculin in focal adhesions at the cell periphery. Moreover, immunoprecipitation with Cdk5 antibody and GST pull-downs with GST-Cdk5 indicated that Cdk5 was part of a protein complex containing vinculin in spreading HCLE cells. To examine the possible function of Cdk5 in focal adhesions, endogenous expression of Cdk5 was suppressed by transient transfection with an siCdk5 plasmid. Immunofluorescence showed a significant reduction of vinculin-containing focal adhesions in siCdk5 transfected cells as compared with cells transfected with a scrambled control ($p<0.001$). Western blot analysis of transiently transfected cells revealed that siCdk5 also decreased Src autophosphorylation at Y416 and Src-dependent phosphorylation of FAK at Y576, but increased total FAK expression and FAK autophosphorylation at Y397. Since FAK autophosphorylation is a sign of integrin activation, these findings suggest that siCdk5 arrests focal adhesion formation prior to Src activation without blocking integrin engagement and activation. Together these findings indicate that Cdk5 is an essential component of focal adhesions, which may play a role in recruitment of activated Src to nascent adhesion sites in spreading corneal epithelial cells.

151/B93

Anchoring of Vinculin to the Lipid Membrane Influences Its Tyrosine Phosphorylation on Position 1065.

G. Diez[1], P. Kollmannsberger[1], A. A. Krukiewicz[1], T. M. Koch[1], C. T. Mierke[1], H. Vali[2], B. Fabry[1], W. H. Goldmann[1]; [1]Center for Medical Physics and Technology, Friedrich Alexander University, Erlangen-Nuremberg, Erlangen, Germany, [2]Department of Anatomy and Cell Biology, McGill University, Montreal, QC, Canada

The focal adhesion (FA) protein vinculin (1066 residues) can be separated into a 30kDa head and a 95kDa tail domain. Vinculin's lipid binding sites localized on the tail, helix 3 (residues 944-978) and the unstructured C-terminus (residues 1052-1066, so called lipid anchor), are important for cell migration and adhesion. It was demonstrated in vivo that vinculin deficient in membrane binding still localizes in focal adhesion contacts. These contacts were more stabile compared to cells with vinculin wildtype protein. We have characterized the mechanical influence of vinculin's lipid anchor in MEFvin(-/-) cells transfected with EGFP-linked-vinΔC (residue 1-1052) using the magnetic tweezer. MEFvinΔC cells were less stiff and more beads detached during these measurements compared to MEF-rescue cells. 2D traction- and confocal microscopy also showed that MEFvinΔC cells generate less force and form fewer FA contacts. However, 2D-traction measurements, using vinculin with mutations in helix 3, which resulted in impaired lipid binding, showed no effect on force generation. Mutating only the c-src phosphorylation site Y1065 on vinculin to phenylalanine, whilst keeping the lipid binding region intact, gave similar

tractions to MEFvinΔC cells. This suggests that membrane interaction of the lipid anchor affects cell mechanical behavior via tyrosine 1065 phosphorylation.

152/B94

A Mouse FAK Mutant Unable to Undergo Autophosphorylation Allows Parsing FAK Functions In Vivo.

J. M. Corsi[1,2], C. Houbron[3,4,5], P. Billuart[3,4,5], J. A. Girault[1,2], H. Enslen[1,2]; [1]Institut du Fer-à-Moulin, UMR-S839, Inserm, Paris, France, [2]UPMC, Paris, France, [3]Plateforme de recombinaison homologue, Institut Cochin, UMR-S567, Inserm, Paris, France, [4]UMR8104, CNRS, Paris, France, [5]Université Paris V René Descartes, Paris, France

Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase critical for processes ranging from embryo development to cancer progression. In mice, FAK null mutation is lethal at embryonic day (E) 8.5 and studies with conditional FAK-deleted mice demonstrated that it plays a critical role in the nervous system, in angiogenesis and in cardiac development. Autophosphorylation of FAK on Tyr-397 is a critical event, allowing, binding of Src family kinases and activation of signal transduction pathways. Despite its importance, the role of FAK autophosphorylation on Tyr-397 has not been investigated in vivo. We addressed this question using a mutant mouse deleted of FAK exon 15 (FAKD/D), which encodes 19 amino acids including Tyr-397. The FAK mutant resulting from this deletion (FAKD) is an active kinase, stably expressed at a physiological level in homozygous mutant embryos. Importantly, FAKD/D embryos have no developmental abnormalities until embryonic day E13.5 indicating that FAK autophosphorylation is not required for normal development up to that stage. This result contrasts with the early lethality at E8.5 of FAK null embryos. However starting at E13.5-E14.5, FAKD/D embryos show vascularization defects and developmental retardation correlated with increased embryonic lethality. The vast majority of FAKD/D embryos does not survive past E16.5 (98.5%) suggesting that FAK autophosphorylation is critical to pursue normal development past E14.5. Interestingly a few FAKD/D embryos (1.5%) were able to develop normally to adulthood demonstrating that the defect due to FAKD expression can be overcome permanently. We further characterized the roles of FAK autophosphorylation in multiple molecular interactions and cellular processes in which FAK had been implicated using primary fibroblasts (MEFs) established from FAKD/D embryos. Most notably we tested in these cells the effects of FAK exon 15 deletion in response to integrin engagement. Our results demonstrate that the physiological functions of FAK in vivo are achieved through both autophosphorylation-independent and autophosphorylation-dependent mechanisms.

153/B95

Utilizing the Delivery of Small Interfering RNA (siRNA) by Microinjection to Demonstrate the Requirement of Focal Adhesion Kinase (FAK) for Bovine Fertilization and Activation.

B. R. Sessions, A. H. Bayles, J. Collier, A. P. Davis, K. L. White; Department of Animal, Dairy, and Veterinary Sciences and Center for Integrated Biosystems, Utah State University, Logan, UT

Focal adhesion kinase (FAK), also known as protein tyrosine kinase 2, is localized to focal adhesions and is a key component of signal transduction pathways mediated by integrins. The clustering of integrins at focal contacts results in activation of FAK and the subsequent assembly of signaling complexes including SRC family kinases. Because integrins have been implicated in bovine fertilization and activation (Campbell et al., 2000; Sessions et al., 2006), the investigation of the involvement of FAK is an essential step for elucidating the molecular mechanisms involved in bovine fertilization and activation. Small interfering RNA (siRNA) directed against the bovine FAK mRNA sequence was microinjected into bovine oocytes and the subsequent effects on FAK mRNA levels and bovine fertilization were evaluated. Real time PCR (qPCR) was used to quantify the levels of FAK message present in bovine oocytes at the time of injection (18 hrs post maturation) and 6, 12, and 24 hrs post injection. The qPCR results indicated a near complete knockdown of FAK levels in bovine oocytes only 6 hours post injection with the levels of FAK in microinjected oocytes increasing at 12 and 24 hrs post injection; however, the FAK levels of the microinjected oocytes never reached the levels present in control oocytes. Oocytes microinjected with FAK siRNA were also fertilized (IVF) and cultured in vitro according to our standard laboratory procedures (Reed et al., 1996). The oocytes microinjected with FAK siRNA had significantly lower cleavage rates compared to the oocytes injected with a negative control siRNA, non-injected, and sham injected treatment groups. These data illustrate a critical involvement of FAK in bovine fertilization and activation; further implicating the involvement of integrins in bovine fertilization.

154/B96

Molecular Mechanisms of Vinculin Activation.

J. Golji, M. Mofrad; Bioengineering, UC Berkeley, Berkeley, CA

Vinculin plays a key role in formation of both focal adhesions and adherens junctions. In focal adhesions vinculin links the integrin bound talin molecule to the cytoskeletal actin filaments. In adherens junctions vinculin reinforces crosslinking of actin filaments by α-actinin. Cytoplasmic vinculin molecules are in an inactive autoinhibitory conformation, while vinculin molecules at both focal adhesions and adherens junctions are in an activated conformation. Vinculin undergoes a conformational change and becomes activated only at those regions that are in need of cytoskeletal reinforcement. The objective of this study is to determine the structural and molecular mechanisms underlying the conformational shift from the autoinhibitory conformation to an active conformation. In the study we used molecular dynamics simulations and normal mode analysis to explore vinculin's possible conformational changes. The simulations demonstrate that vinculin activation requires conformational changes in domain 1 (D1) of the vinculin head (Vh). Movement in D1 removes steric hindrance preventing complete association of the vinculin tail (Vt) with filamentous actin (F-actin). Fifteen salt bridges stabilize the Vt-Vh autoinhibitory conformation. Activation is achieved by breaking 3 of the 15 electrostatic side group interactions. Molecular dynamics show that an external force of under 200pN can lead to conformational change from the autoinhibitory conformation to an activated vinculin conformation, suggesting a role for mechanical force in activating vinculin. Normal mode analysis also revealed movement in D1 of Vh to be a natural movement of the molecule. In conclusion, the molecular dynamics simulations and normal mode analysis have demonstrated that vinculin activation requires conformational changes in D1 of Vh and not complete dissociation of Vt from Vh. Furthermore, tethering of the vinculin to talin at D1 exposes vinculin to an external force that can drive vinculin activation.

155/B97

ROCKII Activity Regulates Tumor Cell Adhesion.

J. Vitko, A. Struckhoff, C. Liu, K. Foderingham, C. Davis, B. Worthylake; Pharmacology, LSU Health Sciences Center, New Orleans, LA

ROCK is a serine/threonine kinase activated by the small GTPase, RhoA which is associated with cell invasion and metastasis. Because of its strong positive effect on integrin adhesions, we hypothesize that one mechanism for promoting invasion and metastasis is by stimulating adhesion between circulating tumor cells with the vascular endothelium of distal organs as the first step in extravasation. Our experimental investigates the effect of manipulating ROCK activity in a system designed to mimic the initial adhesion step during extravasation. Interestingly, our results unexpectedly showed an increase in cellular adhesion at early time points when ROCK activity was inhibited with Y-27632. Because cell adhesion events are directly related to the formation of individual adhesion complexes, we used TIRF microscopy to observe integrin adhesion complexes containing GFP-paxillin during the first 20 minutes of adhesion. In control cells, we observed the formation of numerous adhesion complexes resembling those typically found in adherent cells. In cells treated with Y-27632, adhesion complexes still formed, but were disorganized compared to controls. This demonstrates that while abnormal, the adhesion complexes formed in the absence of ROCK activity retained the ability to support cellular adhesion. We predict that during initial cell adhesion events, Rho and ROCK activity are transiently decreased to promote the transition from suspension to adhesion. After initial adhesion, we expect Rho and ROCK activity will be activated to promote appropriate regulation of adhesion complexes necessary for cell migration and invasion. To test the requirement for an initial inhibition of ROCK activity during cell adhesion, we blocked any decrease in ROCK activity by expressing a constitutively active ROCKII mutant. Cells expressing constitutively active ROCKII effectively blocked cell adhesion, supporting the model that ROCK activity must be decreased to promote early cell adhesion events.

156/B98

Dual Regulation of Dynamin2 Function at Focal Adhesions by Src Kinase.

Y. Wang[1], H. Cao[2], M. McNiven[1,2]; [1]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, [2]Miles and Shirley Fiterman Center for Digestive Diseases, Mayo Clinic, Rochester, MN

Focal adhesions (FAs) are known to play an important role in tumor cell migration, although the mechanisms that regulate the turnover of these structures are poorly understood. The large GTPase Dynamin2 (Dyn2), with an established role in endocytosis, has been identified at FAs and is required for microtubule-induced FA disassembly, but how Dyn2 is recruited to FAs and whether it regulates FA dynamics is not clear. We have observed that activation of Src kinase either through expression of a constitutively active Src mutant SrcY530F or EGF stimulation markedly increases recruitment of Dyn2 to FAs. Conversely, expression of a Src SH2 domain mutant (R178L) that does not localize at FAs, or treatment with the Src kinase inhibitor PP2, prevents the targeting of Dyn2 to FAs. To define how Src activity might induce the recruitment of Dyn2, we first tested the role of the adaptor protein Grb2 which binds to the PRD domain of Dyn2. Interestingly, Grb2 was also recruited to FAs following Src activation, and knockdown of Grb2 inhibited Dyn2's FA targeting. As Grb2 is known to bind to focal adhesion kinase (FAK) on a Src phosphorylation site Y925, we predict that phosphorylation of this Tyr by Src induces the recruitment of Grb2-Dyn2 to FAs. Second, Dyn2 is phosphorylated on Tyr 231, 597 following Src activation, and we tested if this altered FA dynamics and function. We compared the cellular effects of a phospho-mimic mutant Dyn2 Y231, 597E to a non-phosphorylatable form Dyn2 Y231, 597F on FA turnover and cell motility. Interestingly, expression of the Dyn2 YE form greatly increased internalization of β1-integrin compared to WT cells. Further these mutant cells became rounded and poorly adherent with more dynamic FAs. Together, these findings suggest that Src kinase acts to regulate FA dynamics in two related but distinct ways. First, by increasing Dyn2 recruitment to adhesion sites via FAK/Grb2, and second, via phospho-activation of Dyn2 leading to increased endocytic turnover of FA components.

157/B99

The Rap1 GTPase Alters Focal Adhesion Dynamics by Resetting the ECM Ligand Density 'Sensor'.

S. A. Freeman[1,3,4], S. McLeod[1,2,3], J. Dukowski[1,2], M. R. Gold[2,3,4], C. D. Roskelley[1,3,4]; [1]Cellular and Phyiological Sciences, University of British Columbia, Vancouver, BC, Canada, [2]Department of Microbiology and Immunology, University of British Columbia, Vancouver, BC, Canada, [3]CELL Research Group, University of British Columbia, Vancouver, BC, Canada, [4]I3 Research Group, University of British Columbia, Vancouver, BC, Canada

Productive cell migration, which is an important component of cancer metastasis, requires cells to sense changes in the extracellular matrix (ECM). Using fibronectin as a planar ECM ligand for metastatic B16 melanoma cells, we show that increasing the ligand density first increases and then decreases productive, polarity-dependent cell migration. These changes in migration were associated with alterations in focal adhesion dynamics that were represented by a gradual increase in the mobile fraction of paxillin and the focal adhesion kinase as determined by fluorescence recovery after photobleaching. In contrast, the mobility of the α5 integrin initially increased at low ECM ligand densities when cells were motile but then decreased dramatically at high ECM ligand densities when motility slowed. Thus, we conclude that a decrease in integrin subunit mobility at focal adhesions is a read-out of a putative cellular ECM ligand density 'sensor'. As the Rap1 GTPase stimulates integrin binding to ECM ligands, we reasoned that it might reset this sensor. Indeed, elevated Rap1-GTP decreased α5 integrin mobility at low ECM ligand densities and this was dependent, at least in part, on the downstream effector RapL. As a result, Rap1-GTP increased focal adhesion stability and number such that cell polarity and motility were reduced under conditions of low ECM ligand density that normally stimulate productive migration. Furthermore, this apparent Rap1-mediated resetting of the sensor had biological consequences consistent with a decrease in motility. Specifically, it greatly reduced the post-adhesive migration of the B16 melanoma cells across a vascular endothelial monolayer. The latter effect likely contributed to the decrease in metastatic lung colonization we observed in vivo when Rap1-GTP was chronically elevated in these cells.

158/B100

Talin as a Mediator of Prostate Cancer Cell Migration, Invasion, and Metastasis.

S. Sakamoto[3], R. O. McCann[1,2], N. Kyprianou[3,2]; [1]Basic Medical Sciences, Mercer University School of Medicine, Macon, GA, [2]Molecular and Cellular Biochemistry, University of Kentucky, Lexington, KY, [3]Surgery: Division of Urology, University of Kentucky, Lexington, KY

Talin1 is an integrin binding protein that regulates integrin activation and mediates signaling interactions with the extracellular matrix (ECM). The role of talin in human cancer metastasis has not been examined in depth. In this study we have investigated the functional significance of this key focal adhesion component in the regulation of prostate cancer cell invasion in the context of the tumor microenvironment and metastatic behavior in vitro and in vivo using prostate cancer cells from the TRAMP mouse model of prostate adenocarcinoma. Our results demonstrate that Talin1 overexpression correlated with prostate cancer development and progression to metastasis in the TRAMP mouse model. Stable expression of Talin1 in PC-3 prostate cancer cells enhanced cell adhesion and migration, and promoted invasion prostate cancer cells in vitro. shRNA-mediated silencing of Talin1 resulted in a significant suppression of the invasion potential of prostate cancer cells. Mechanistic dissection of signal transduction in this model revealed that Talin1 regulates cell survival signals through activation/phosphorylation of focal adhesion kinase (FAK) and AKT. These findings identified talin as a potent mediator of prostate tumor cell migration, invasion and intracellular survival signaling. Thus, talin emerges as a potential therapeutic target for advanced prostate cancer, as well as a tumor marker that might predict aggressive prostate tumors during progression to metastasis.

159/B101

Analysis of Traction Stress Variation Across Single Focal Adhesions.

S. V. Plotnikov[1], B. Sabass[2], U. S. Schwarz[2], C. M. Waterman[1]; [1]Laboratory of Cell and Tissue Morphodynamics, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD, [2]University of Heidelberg, Heidelberg, Germany

The ability of eukaryotic cells to generate force and sense the mechanical properties of the extracellular matrix (ECM) underlies many biological processes, such as cell migration, proliferation and differentiation. This is achieved in part by integrin-mediated focal adhesions (FA), protein assemblies that couple contractile actomyosin bundles to the ECM and transmit force generated by the cytoskeleton across the plasma membrane. It has been recently demonstrated that protein composition and/or post-translational modification state can vary across an individual FA. However, whether this translates to variation in physiological properties and/or function for sub-FA domains is not known. We hypothesize that variation in biochemical composition across an individual FA mediates differences in force transmission across a FA that may be critical to it role in cell migration. To test this, we used high-resolution traction force microscopy (Sabass et al., 2008) to analyze the distribution of traction stresses along single FA and correlate this with the density of specific FA proteins. We identified two distinctive patterns of traction stress: for the majority of FAs, the region of maximum stress was located to the geometrical center of the FA, while for some FA it was significantly skewed toward their distal edge (cell periphery). Translocation of the traction force maximum from the FA center towards its distal edge usually occurred shortly before cell tail retraction. Analysis of GFP-paxillin intensity in FA revealed that maximum traction stress colocalized with maximum paxillin concentration, independent of the position of maximal stress within the FA. Next, we tested the hypothesis that mechanical stress applied on FA can induce translocation of the traction stress maxima. We found that centripetal pulling on a fibronectin-coated magnetic bead attached to the dorsal side of the cell caused displacement of traction force in ventral FA similar to that observed in FA in a retracting cell edge. These results suggest that FA biochemical and mechanical properties are directly coupled. *The first two authors contributed equally to this work.

160/B102

Three Dimensional Superresolution Fluorescence Microscopy Reveals Protein Stratification in Focal Adhesions.

P. Kanchanawong[1], G. Shtengel[2], M. W. Davidson[3], H. Hess[2], C. M. Waterman[1]; [1]Laboratory of Cell and Tissue Morphodynamics, National Heart Lung and Blood Institute, Bethesda, MD, [2]Janelia Farm Research Campus, Howard Hughes Medical Institute, Ashburn, VA, [3]National High Magnetic Field Laboratory and Department of Biological Sciences, Florida State University, Tallahassee, FL

Focal Adhesions (FA) are dynamic structures consisting of large numbers (>150) of different proteins that mechanically link the actin cytoskeleton to the extracellular matrix (ECM). Despite the central role of FA in cell migration and the wealth of biochemical and cell biological data on FA proteins, it remains virtually unknown how these proteins are organized within FA. Based on the differential dynamics of distinct FA proteins we previously observed using fluorescent speckle microscopy, we hypothesized that FA proteins may be organized into stratified layers within FA that serve as dissipative elements in a "molecular clutch" to form a regulatable, force-transducing link between the actin cytoskeleton and the ECM. To test this hypothesis, we employed a 3-dimensional superresolution fluorescence microscopy technique, interferometric photoactivated localization microscopy (iPALM), to determine sub-20 nm z-axis localizations of several key structural components of FA labeled with photoactivatable fluorescent proteins and expressed in U2OS cells plated on a fibronectin-coated substrate. Farnesylated tdEosFP was used to demarcate the cytoplasmic face of the plasma membrane and was localized at ~20 nm above the substrate. Paxillin, talin, and vinculin were distinguished by distinct vertical distributions, with the highest densities at 25, ~30, and ~50 nm above the substrate plane, respectively. Paxillin and talin formed narrow distributions with FWHM <15 nm, while the vinculin layer was broader with FWHM ~25 nm. Actin appeared to be largely excluded from the bottom layers of FA up to a height of ~50 nm, with the highest density observed at ~90 nm above the substrate plane. iPALM reveals for the first time that different FA proteins are segregated into distinct layers parallel to the substrate plane. The protein stratifications in FA provide a structural context for the mechanosensing and mechanotransducing functions of FA.

161/B103

Proteomics Analysis of Focal Adhesion Maturation.

J. Kuo[1], X. Han[2], J. R. Yates[2], C. M. Waterman[1]; [1]NHLBI/NIH, Bethesda, MD, [2]Cell Biology, Scripps Research Institute, La Jolla, CA

Focal adhesions (FA) are complex plasma-membrane associated macromolecular assemblies that serve to physically connect cells to, as well as transducer signals to and from, the surrounding extracellular matrix (ECM). FA play a crucial role in the control of tissue structure and morphogenesis as well as cell motility. It is well established that FA undergo a "maturation" process in which their size increases that is regulated by either small GTPase signaling or by force applied to the FA. Indeed, Rac1 signalling or reduced force promotes formation of small FA, while RhoA signalling or high force promotes FA growth and maturation. The maturation of FA is thought to be critical to modulating the signals transduced from the ECM into the cell that regulate cell growth and differentiation. However, how FA maturation achieves signal modulation is not known. Here, we test the hypothesis that FA maturation alters FA protein composition, and further, that

physical and biochemical pathways to FA maturation may not necessarily be the same. We developed a systematic method for biochemical isolation of focal adhesions from human fibroblasts, followed by analysis of the FA protein compositions by the Mud-pit LC-MS proteomics method. To test our hypothesis, we modulated FA maturation in several ways and analyzed FA protein composition. To alter the force on FA, we either treated cells with the myosin II inhibitor blebbistatin or cultured cells on polyacrylamide sustrates of different stiffnesses. To alter FA maturation biochemically, we expressed constitutively active mutants of RhoA (V14) or Rac1 (V12). This study provides a global view how FA composition is altered by physical and biochemical cues, and provides insight into how physical signals alter focal adhesion-related signaling networks to trigger diverse physiological functions.

Membrane Fusion (162-172)

162/B105

Electrostatic Interactions between VAMP2 and Acidic Phospholipids Are Required for Membrane Fusion.

D. Williams[1], J. Vicogne[2], I. Zaitseva[3], S. McLaughlin[3], J. E. Pessin[1]; [1]Department of Medicine, Division of Endocrinology, Albert Einstein College of Medicine, Bronx, NY, [2]Institut de Biologie de Lille, Lille Cedex, France, [3]Physiology & Biophysics, Stony Brook University, Stony Brook, NY

Intracellular membrane transport requires assembly of SNARE proteins to promote fusion between donor and acceptor membranes. The R-SNARE protein VAMP2 contains a highly conserved juxtamembrane domain with tandem tryptophans flanked by clusters of basic residues. We demonstrate that this domain binds acidic phospholipids through direct electrostatic interactions that increased the partitioning of the tandem tryptophan residues into the phospholipids bilayer. Neutralization of the membrane electrostatic potential with amphipathic bases resulted in the partitioning of the tryptophan residues out of the phospholipid bilayer and inhibited binding of the VAMP2 basic cluster. Moreover, reduction of the electrostatic potential of acidic phospholipid membranes in vivo inhibited calcium-stimulated insulin secretion in beta cells and GLUT4 vesicle fusion in adipocytes. Consistent with these results, expression of VAMP2 proteins containing mutations that neutralize these juxtamembrane positive charges also inhibited plasma membrane fusion of GLUT4-containing vesicles in adipocytes. Together, these data support a general model of protein-lipid interaction in which the positively charged VAMP juxtamembrane regions facilitate fusion by bridging the negatively charged plasma and vesicle membrane leaflets.

163/B106

SNARE Specificity and Availability: How They Regulate Intracellular Fusion.

I. Bethani[1], C. Kadian[2], T. Lang[3], R. Jahn[1], S. O. Rizzoli[4]; [1]Neurobiology, Max Planck Institute for biophysical Chemistry, Göttingen, Germany, [2]International Max Planck Research School, Göttingen, Germany, [3]Molecular Immune and Cell Biology Unit, LIMES Institute, Bonn, Germany, [4]European Neuroscience Institute, Göttingen, Germany

Soluble N-ethylmaleimide-sensitive factor attachment receptor (SNARE) proteins catalyze organelle fusion in the secretory pathway, and different fusion steps, involving different processes and organelles, are mediated by specific SNARE sets. However, the mechanisms ensuring that only members of the same SNARE set (cognate SNAREs) interact with each other for fusion remain unclear. By studying the SNAREs residing on the membrane of early endosomes, we have investigated how the specificity of SNARE pairing and function is regulated (Bethani et al., EMBO J., 2007). Early endosomal and exocytic SNAREs colocalize on the early endosomal membrane and associate in promiscuous cis-complexes, in a mass-action-dependent manner, but they show no crosstalk regarding their functions (early endosomal fusion and exocytosis, respectively). By applying a variety of biochemical and microscopic techniques, we have demonstrated that two synergistic mechanisms ensure specificity in fusion: (i) the enrichment of the necessary, cognate SNAREs at the prospective fusion sites and the exclusion of the non-cognate ones, and (ii) the preference for cognate SNARE associations in trans. Considering the importance of intracellular fusion for cell viability, we further asked how robust fusion-regulating mechanisms are. To challenge the system, we used siRNA techniques to downregulate the amount of single early endosomal or exocytic SNAREs. We have shown that the cellular fusion machinery is not affected by silencing of individual SNAREs and that surprisingly, the residual SNARE levels present in the knockdown cells seem to be enough to sustain fusion functionality. While SNAREs are organised in membrane domains, they follow a more diffused pattern upon downregulation of their expression. In this way, they remain available to levels similar to the wild-type. We therefore suggest that SNAREs are provided in enough abundance to prevent any malfunction of intracellular fusion and that the co-operative regulation of their functional specificity and availability ensures intracellular fusion.

164/B107

Catch-and-release of Prefusion trans-SNARE Complexes on Intact Organelles.

M. L. Schwartz, A. J. Merz; Biochemistry, University of Washington, Seattle, WA

To catalyze membrane fusion, eukaryotic SNARE proteins embedded in opposite lipid bilayers assemble into tight trans-complexes, a process that is thought to drive the membranes into close apposition. Trans-SNARE complexes are hypothesized to nucleate at the membrane-distal SNARE N-termini and "zipper" toward the membrane-proximal C-termini. However, the functional relationship between SNARE assembly and fusion remains poorly defined, particularly on native membranes. We now employ lipid and content mixing assays with intact organelles to dissect the roles of C-terminal zippering in trans-SNARE complex assembly and fusion catalysis. Our results show that partially-zipped trans-SNARE complexes can persist on docked membranes in the absence of lipid or content mixing, and that C-terminal zippering of the SNARE complex triggers fusion by initiating lipid mixing. Remarkably, the fusion competence of a partially assembled, fusion-defective trans-complex can be rescued by the universal SNARE chaperone Sec17/α-SNAP. Sec17-mediated rescue occurs with steep cooperativity, indicating the involvement of multiple trans-complexes, and without Sec18/NSF activity. Together, these experiments uncover distinct functionalities within the C-terminal region of the SNARE complex and demonstrate that extrinsic stabilization of a partially assembled trans-SNARE complex can trigger fusion. The methodology developed in these studies will facilitate detailed in situ analyses of prefusion intermediates on native membranes.

165/B108

A Role for Lipid Flippases in Vacuole Microdomain Assembly and Membrane Fusion.

T. Sasser, R. Fratti; Biochemistry, University of Illinois at Urbana-Champaign, Urbana, IL

Vacuole fusion occurs at a specialized microdomain that forms upon membrane docking. When vacuoles dock, membranes in contact become drawn together and form two tightly apposed flattened disks called the boundary domain. The edge of the boundary, where membranes meet, is the vertex ring. The vertex ring is the site of fusion, where SNAREs, Rab GTPases, tethering factors, and regulatory lipids (e.g. phosphoinositides) accumulate and mediate fusion. Although the vertex ring has been significantly characterized, the role of the boundary membrane remains unclear. We hypothesized that membrane deformation at the boundary is driven by membrane remodeling through lipid metabolism and/or lipid translocation between leaflets. The translocation of phospholipids across bilayers is mediated by a group of proteins termed flippases, which include class C ABC transporters. Saccharomyces cerevisiae vacuoles contain three class C ABC transporters named Ycf1p, Bpt1p and Ybt1p. Ycf1p is enriched at the boundary membrane. Vacuoles isolated from ycf1Δ strains possessed elevated levels of the fusion-regulating lipid Phosphatidylinositol 3-phosphate (PI3P). Moreover, membrane fusion was inhibited in vacuoles deleted in both ycf1Δ and bpt1Δ. These results were recapitulated by treatment of wild type vacuoles with the compound MK571, an inhibitor of ABC transporters that blocks molecule translocation across bilayers. Because Ycf1p interacts with the PI3P 5-kinase Fab1p, it is likely that PI3P is converted to PI(3,5)P2 during fusion, and that in the absence of Ycf1p, Fab1p is either missorted or non-functional, leading to an accumulation of PI3P and dysregulated fusion. It is also possible that Ycf1p equilibrates PI or PI3P across the bilayer. Therefore, ycf1Δ vacuoles retain more PI substrate, and thus more PI3P on the cytosolic face of the membrane. These data illustrate that ABC transporters have a direct role in phosphoinositide metabolism and membrane fusion.

166/B109

Minimal Requirements for Rab GTPase-mediated Inter-membrane Interactions Revealed by Reconstitution of Rab GTPase-dependent Lipid Mixing from Purified Components.

C. Stroupe, J. Mima, C. M. Hickey, A. S. Burfeind, W. T. Wickner; Department of Biochemistry, Dartmouth Medical School, Hanover, NH

Rab GTPases are central regulators of many intracellular trafficking events, but understanding of the biochemical mechanisms underlying their activities has been hampered by a scarcity of chemically defined systems for studying their action. We now report a novel reconstituted membrane fusion system using proteoliposomes bearing the yeast vacuolar Rab GTPase Ypt7 purified in its active, geranylgeranylated form. Mixing of the lipids in the membranes of these proteoliposomes requires Ypt7 and its purified effector, the HOPS/Class C Vps complex, in addition to vacuolar SNAREs and the SNARE chaperones Sec17 and Sec18. This lipid mixing occurs without detectable lysis. Ypt7 must be in its GTP-bound form for lipid mixing to take place. Clustering of proteoliposomes requires not only Ypt7 and HOPS complex but also SNAREs, suggesting that Ypt7p-HOPS complex interactions are insufficient for membrane tethering. This is the first system for reconstitution of membrane fusion that requires both a purified Rab GTPase and its purified effector, and it thus offers unprecedented opportunities to investigate fundamental biochemical mechanisms of Rab GTPase-, Rab effector-, and SNARE-dependent membrane fusion.

167/B110

Components of the Bacteriophage P22 Infection Machinery at Subnanometer Resolution.

G. C. Lander[1], A. Evilevitch[2], M. Jeembaeva[2], C. S. Potter[1], B. Carragher[1], J. E. Johnson[1]; [1]Cell Bio, The Scripps Research Institute, La Jolla, CA, [2]Biochemistry, Lund University, Lund, Sweden

The bacteriophage P22 tail machine is a multifunctional protein structure that is built from 51 subunits of five different gene products. These components, necessary for phage assembly, adsorption and infection, are organized through a combination of 3-, 6-, and 12-fold symmetry. Here we present the 3-dimensional structure of the isolated P22 tail machine as determined by single particle cryo-electron microscopy, whose resolution extends beyond previously published work. We have additionally solved the structure of the isolated portal component of the structure in its 11 and 12-fold oligomeric state. The gene product delineations that were proposed in the previous isolated tail machine reconstruction and asymmetric virion reconstruction are much more well-defined here, such that a much more thorough proposal of the gene product constitution is possible. The detail provided by this reconstruction provides an insightful look at the subunit-subunit interactions that make up the tail machine, as well as into the molecular events that occur during host cell attachment and DNA translocation.

168/B111

Contribution of the Vacuolar H+-ATPase to Different Steps of Membrane Fusion Analyzed In Vitro and In Vivo.

B. Strasser; Biochemistry, University of Lausanne, Epalinges, Switzerland

The membrane integral V0 sector of the vacuolar H+-ATPase (V-ATPase) is necessary for membrane fusion between yeast vacuoles (lysosomes) (Peters, 2001; Bayer, 2003; Baars, 2007), for secretion of neurotransmitters and insulin, phagosome-lysosome fusion and the secretion of multivesicular bodies. (Hiesinger 2005; Peri, 2008; Liegois, 2006; Sun-Wada, 2008). The goal of the presented project is to investigate how V0 contributes to fusion and how it interacts with SNAREs. We utilize random mutagenesis to identify sites in V0 subunits that are selectively involved in vacuole fusion but dispensable for the proton pump function of the V-ATPase. Yeast cells were subjected to random mutagenesis and screened in vivo to identify mutant variants of V0 subunits that still function in proton pumping but cause fragmented vacuoles, indicative of a fusion defect. Fusion characteristics of selected mutants were analyzed in vitro with purified vacuoles (Reese et al., 2005). Mutants with selective fusion defects were found in the proteolipids c and c' (Vma3p and Vma11p) and the d subunit (Vma6p). In the proteolipids critical mutations are concentated in the cytosolic half of the transmembrane domains and in the periphery. Content mixing and lipid mixing assays reveal a function of the proteolipid ring in the induction of lipid flow between the membranes as well as in fusion pore opening. Analysis of Vma6p mutants suggest that this subunit may control the priming of SNAREs. The results presented here show that V0 acts in several steps of the fusion process and map these contributions at the subunit or domain level. Mutations found in this screen will be instrumental in disentangling the interactions of the V-ATPase with SNAREs and in defining their contributions to membrane fusion.

169/B112

Pathways of Rab GTPase Activation and Inactivation at the Yeast Vacuole.

A. J. Merz, C. L. Brett; Biochemistry, University of Washington, Seattle, WA

Rab GTPases are key regulators of membrane docking and fusion, but despite intense biochemical study, the in vivo pathways leading to activation and inactivation of most Rabs are poorly characterized. We present a global analysis of the effects of Rab GAP overexpression on yeast vacuole morphology. Overproduction of three GAPs, Gyp3, Gyp4, and Gyp7, cause vacuole fragmentation, consistent with a membrane fusion defect. We show that Gyp7 inactivates the yeast vacuole Rab Ypt7 in vivo. However, to efficiently terminate Ypt7 signaling, Gyp7 requires downstream assistance from a casein kinase I, Yck3. Yck3 mediates phosphorylation of at least two targets of Ypt7 signaling: a tethering factor, Vps41, and a SNARE, Vam3. Yck3-dependent phosphorylation of both Vps41 and Vam3 is inhibited by Ypt7:GTP. We also show that Ypt7 binds Vps41 directly, and that Vps41 is essential for stable HOPS interactions with Ypt7. Together, these results indicate that Gyp7-stimulated GTP hydrolysis on Ypt7 triggers both passive termination of Ypt7 signaling and active, kinase-mediated shut-off of downstream targets. We propose a model incorporating linked feed-forward and feed-back loops that together exert tight control over Ypt7 signaling and membrane fusion.

170/B113

Function of the DENN Domain Revealed through Characterization of Connecdenn.

P. D. Allaire[1], B. Ritter[1], C. Dall' Armi[2], G. Di Paolo[2], P. S. McPherson[1]; [1]Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, QC, Canada, [2]Pathology and Cell Biology, The Taub Institute for Research on Alzheimer's Disease and the Aging Brain, Columbia University Medical Center, New York, NY

The DENN domain is a tripartite module found in multiple proteins involved in vesicle trafficking including DENN/MADD, Sbf1/2, Rab6IP1, and connecdenn. No structural or functional properties have been assigned to the DENN domain. Rab GTPases, a major branch of the Ras superfamily of small molecular weight GTPases, play a key role in the establishment, maintenance and transport of intracellular compartments. Here we demonstrate that the DENN domain of connecdenn functions as a guanine-nucleotide exchange factor (GEF) for Rab35, a GTPase involved in recycling of plasma membrane receptors. Connecdenn is recruited to clathrin-coated vesicles through C-terminal motifs and interacts with lipids through its N-terminal DENN domain. Connecdenn remains stably associated with nascent endocytic vesicles following removal of the clathrin coat, suggesting a post endocytic function. In fact, neither connecdenn nor Rab35 knock down affects endocytosis of the epidermal growth factor or transferrin receptors but strongly disrupts their intracellular trafficking. Furthermore, inactivation of Rab35 by knock down of either Rab35 or connecdenn leads to a dramatic enhancement of neurite outgrowth in PC12 cells upon NGF stimulation, indicating a perpetuation of endosomal signaling. Altogether, we demonstrate the first functional activity of a DENN domain and position connecdenn with Rab35 in trafficking early in the endosomal pathway. Moreover, our results lay the cornerstone to better understand the function of DENN domain-containing proteins implicated in human disease including MADD/DENN (Alzheimer's), Sbf1/2 (Charcot-Marie-Tooth) and ST5 (cancer).

171/B114

Rab-mediated Membrane Tethering with Purified Components.

S. L. Lo[2,1], C. L. Brett[1], A. J. Merz[1]; [1]Biochemistry, University of Washington, Seattle, WA, [2]Chemistry, University of Washington, Seattle, WA

Rab GTPases are implicated in membrane tethering and docking reactions at many different endomembrane vesicular transport steps. However, the mechanisms by which activated Rabs promote tethering remain largely opaque. Using purified lipids and proteins, we have reconstituted membrane tethering in a reaction that depends on GTP-bound Vps21, the *Saccharomyces cerevisiae* paralog of mammalian Rab5, which promotes tethering of liposomes. We present two quantitative assays for tethering, one of which allows evaluation of both heterotypic and homotypic tethering events, and we demonstrate the reversibility of tethering when Vps21 is inactivated by the Rab GAP Gyp1. This system provides an experimental framework for ground-up biochemical reconstitution of the upstream pathways that control Vps21 signaling and endosome tethering.

172/B115

Intracellular Sterol Transport Controls Vacuole Fusion and Fission during Cellular Aging.

F. Tang[1], P. Chandler[1], A. M. Sturm[2], M. Bard[2]; [1]Biology, University of Arkansas at Little Rock, Little Rock, AR, [2]Biology, IUPUI, Indianapolis, IN

During each round of the yeast cell cycle, vacuoles divide and fuse. We recently discovered that aged yeast cells have large round vacuoles, an indication of a defect in vacuole division or fission. Additionally, mutants with large round vacuoles have a shortened life span. However, mutants (erg6Δ, nyv1Δ, etc) defective in vacuolar fusion also have a shortened life span especially under caloric restriction (Tang et al., 2008. Autophagy 4(7)). To further explore the mechanisms of vacuolar dynamics in aging, we analyzed the sterol profile of erg6Δ cells in different media and found that caloric restriction up-regulated the products of the enzyme Erg2. Consequently, up-regulation of the expression of ERG2 by the promoter of ERG6 (Perg6-ERG2) extended the yeast life span. Consistent with a role of ergosterol in vacuolar fusion, Perg6-ERG2 partly suppressed the vacuole fusion defect of nyv1Δ. Similar to caloric restriction, Perg6-ERG2 shortened the life span of nyv1Δ. These observations suggested that ergosterol must be depleted from the vacuolar membrane for longevity. A group of proteins that mediate sterol transport among organelles is the oxysterol-binding protein homologs (Osh). Deletion of OSH5 extended yeast life span, suggesting that some form of sterol-transport antagonistic to Osh5 is beneficial to life span extension. To demonstrate such sterol transport pathways, we over-expressed all 7 OSH genes in selected vacuole mutants and found that over-expression of OSH6 promoted vacuole fusion but OSH3 or OSH1 promoted fission in a vacuole mutant atg18Δ, which accumulates elevated levels of phosphatidylinositol 3,5 bisphosphate (PI3,5P2). High levels of PI3,5P2 usually promote vacuole fission. Phosphoinositides are signature lipids of intracellular membranes and can be bound by Osh proteins. Therefore, we hypothesize that some Osh proteins (Osh6) bind PI3P and transport sterols to the vacuolar membrane while some other Osh proteins (Osh3, Osh1) bind PI3,5P2 and remove sterols from the vacuolar membrane. The

incorporation of ergosterol into the vacuolar membrane and the subsequent removal of ergosterol from the vacuolar membrane thus contribute to longevity by controlling vacuole fusion and fission dynamics.

Protein Targeting (173-184)

173/B116

Asna1-mediated Membrane Targeting/Insertion of Tail-anchored Proteins.

V. Favaloro[1], F. Vilardi[1], M. Spasic[2], B. Dobberstein[1]; [1]ZMBH-University of Heidelberg, Heidelberg, Germany, [2]DKFZ-Heidelberg, Heidelberg, Germany

Tail-Anchored (TA) proteins are characterised by a C-terminal transmembrane region that mediates post-translational insertion into the membrane of the endoplasmic reticulum (ER). We have investigated the requirements for membrane insertion of three TA proteins, RAMP4, Sec61β and cytochrome b5. We show that newly synthesised RAMP4 and Sec61β can accumulate in a cytosolic, soluble complex with the ATPase Asna1 before insertion into ER-derived membranes. Membrane insertion of these TA proteins is stimulated by ATP, sensitive to redox conditions and blocked by alkylation of SH groups by N-ethylmaleimide (NEM). By contrast, membrane insertion of cytochrome b5 is not found to be mediated by Asna1, not stimulated by ATP and not affected by NEM or an oxidative environment. We are currently better characterizing the Asna1-mediated route for membrane targeting and insertion of TA proteins.

174/B117

Membrane Recruitment of Human Guanylate-binding Protein 1.

J. Fres[1,2], V. Bade[1,2], S. Müller[3], C. Horst[1,2], G. Praefcke[1,2]; [1]Junior Research Group 6, Center for Molecular Medicine Cologne, Cologne, Germany, [2]Institute for Genetics, University of Cologne, Cologne, Germany, [3]Bioanalytical Laboratory, Center for Molecular Medicine Cologne, Cologne, Germany

Guanylate-binding proteins (GBPs) belong to the dynamin superfamily of large GTPases. They are highly induced by interferon-γ implicating a role in innate immunity. They differ from all other GTPases by their ability to hydrolyze GTP to GDP and GMP. Three of the seven human GBPs carry a C-terminal CaaX box, which serves as a signal sequence for post-translational isoprenylation. Recently, an activation and interferon-dependent recruitment of farnesylated hGBP1 to the Golgi apparatus has been demonstrated. We have analyzed the modification of the endogenous human guanylate-binding protein 1 (hGBP1) isolated from interferon-induced cells. The CTIS sequence leads to attachment of a C15-farnesyl moiety. Subsequently, the TIS peptide is removed by proteolytic cleavage and 70 % of the protein undergo a methylation of the carboxyl group of the C-terminus. In order to study the effect of these modifications we have produced farnesylated and proteolytically processed hGBP1 expressed in S. cerevisiae. The biochemical properties of the modified protein such as nucleotide binding, nucleotide dependent oligomerization and a co-operative GTPase activity differ from the non-modified form. Furthermore, the modified protein displays nucleotide-dependent binding to synthetic liposomes in its activated state with a slight preference for phosphatidylinositol phosphate containing vesicles. Several point mutations which display impaired nucleotide binding and hydrolysis are also defective in liposome binding and activation-dependent recruitment to cellular membranes. Hence, our systems allow us to reconstitute the intracellular dynamics of hGBP1 in vitro and to investigate the mutual regulation of the enzymatic activity and the cellular function.

175/B118

Regulation of Megalin Trafficking by Phosphatidylinositol Metabolism.

S. Cui, C. Guerriero, O. A. Weisz; Univ of Pittsburgh, Pittsburgh, PA

A major function of the proximal tubule of the kidney is reabsorption of low molecular weight proteins from the glomerular filtrate. The multiligand receptor megalin plays an important role in the binding and internalization of these proteins from the apical surface of renal epithelial cells. Defects in normal megalin function result in Fanconi syndrome, a generalized term for pathological conditions in which increased protein is present in the urine. Fanconi syndrome is a hallmark of patients with Lowe syndrome, an X-linked disorder that results from mutations in Ocrl1, an enzyme that hydrolyzes the 5'-phosphate from phosphatidylinositol 4, 5-bisphosphate (PIP2). We hypothesize that PIP2, a versatile player in membrane traffic, regulates biosynthetic and/or endocytic trafficking of megalin in renal epithelial cells. To test this idea, we are manipulating cellular PIP2 levels by siRNA mediated knockdown and overexpression of Ocrl1 and phosphatidylinositol 5-kinase and examining the consequences on megalin surface delivery and endocytosis. This study should further our understanding of how renal Fanconi syndrome develops and may contribute to the development of new therapeutic interventions for Lowe Syndrome.

176/B119

Computational Analyses of Phosphoinositide-binding Domains in Arabidopsis thaliana.

E. Wywial, S. M. Singh; Biology, Brooklyn College CUNY, Brooklyn, NY

Phosphoinositides (PIs) are a family of inositol-containing phospholipids found in all eukaryotic cells, which play critical roles in membrane trafficking and signal transduction. Subcellular targeting of various membrane associated proteins often involves a PI-binding domain. The interactions of these domains with the membrane surface may be highly specific or non-specific, driven by various combinations of contributions from charge, hydrophobic and specific interaction components. Most studies on various aspects of PI-binding proteins have mainly concentrated on mammalian or other non-plant model organisms and these PI-binding domains remain extremely under-investigated even though PIs are known to be critical for various aspects of plant growth and development. In this light we have undertaken a comprehensive analysis of various PI-binding domains in *Arabidopsis thaliana* on a genomic scale using a computational approach that includes a combination of sequence analysis, structure modeling and analysis, ligand docking and examination of biophysical properties. We present the results for the entire family of PH and FYVE proteins encoded in Arabidopsis thaliana genome which reveal domain architectures, binding signatures and specificity, and electrostatic profiles unique to plants. Our results are important in understanding the differences in signaling through PI-binding domains in plants and its implication in plant signaling and membrane trafficking.

177/B120

Lipid Raft Association as a Mechanism for Ciliary/Flagellar Membrane Protein Trafficking.

B. Emmer[1], K. Tyler[2], D. Engman[1]; [1]Pathology, Northwestern University, Chicago, IL, [2]University of East Anglia, Norwich, United Kingdom

Trypanosomes are protozoan parasites that possess a single polarized flagellum. These unicellular organisms have three discrete membrane domains, associated with the pelliculum (cell body), flagellar pocket and flagellum. The molecular determinants of protein and lipid sorting to discrete eukaryotic surface membrane domains, including that of the flagellum/cilium, remain fundamental questions of cell biology. Here, we report that a family of calcium-binding proteins, the calflagins, traffic to the trypanosome flagellum in a manner dependent on N-terminal dual acylation. Unmodified calflagin localizes diffusely throughout the cytoplasm and myristoylated calflagin localizes to the pellicular membrane. Palmitoylation of myristoyl calflagin then leads to proper trafficking of the mature protein to the flagellar membrane. Coincident with this differential sorting upon palmitoylation is an association with lipid raft microdomains, the components of which are highly enriched in the flagellar membrane as determined by biochemical analyses and fluorescence visualization of lipid raft markers and laurdan fluorescence microscopy. Cold detergent extraction and scanning electron microscopy also revealed patches of membrane along the axoneme in a distribution and size reminiscent of intraflagellar transport barges, proteinaceous complexes that transport structural components to the eukaryotic flagellum. Finally, we identified through a reverse genetic screen the specific palmitoyl acyltransferase mediating calflagin palmitoylation and trafficking. Localization of this protein will reveal the branching point at which palmitoylation-dependent trafficking to the flagellar or pellicular membranes diverges.

178/B121

Molecular Coordination of Cotranslational Protein Targeting by the SRP RNA.

N. R. Bradshaw, S. Neher, S. Floor, J. Gross, P. Walter; Biochemistry, University of California, San Francisco, San Francisco, CA

The signal recognition particle (SRP) recognizes ribosomes translating secreted and transmembrane proteins by binding to hydrophobic signal peptides as they are translated. The SRP then interacts with its membrane-associated receptor (SR) to deliver these ribosomes to the membrane. The RNA component of the SRP catalyzes the SRP-SR interaction, accelerating it over 100-fold. We sought to understand the mechanism of SRP RNA catalysis of the SRP-SR interaction and how it contributes to cotranslational protein targeting. We show that truncation of the first helix of the N domain (helix N1) of both the SRP and SR proteins from E. coli dramatically accelerates their interaction. SRP and SR with helix N1 truncations interact at nearly the RNA-catalyzed rate in the absence of RNA. NMR spectroscopy and analysis of GTPase activity show that helix N1 truncation in SR mimics the conformational switch caused by complex formation. These results demonstrate that the N-terminal helices of SRP and SR autoinhibit complex formation in the absence of SRP RNA, suggesting that SRP RNA facilitates the SRP-SR interaction by overcoming this kinetic hurdle. Additionally, we show that synthetic signal peptides activate the SRP RNA to catalyze the SRP-SR interaction. Together, our results provide a unifying model in which a symmetrical conformational switch, using SRP RNA at its core, coordinates the SRP-SRP receptor interaction with SRP cargo recruitment.

179/B122

Localization of Transmembrane Proteins to the TGN.

R. S. Fuller[1], M. Abazeed[1], M. De[1], T. Komiyama[1], A. Hoppe[2], A. Kumar[3,4]; [1]Biological Chemistry, University of Michigan, Ann Arbor, MI, [2]Microbiology and Immunology, University of Michigan, Ann Arbor, MI, [3]Life Sciences Institute, University of Michigan, Ann Arbor, MI, [4]Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI

Transmembrane proteins maintain steady-state localization in the yeast trans Golgi network via cycles of vesicular transport between the TGN and endosomal compartments. We are using cell-free biochemical reconstitution [Abazeed, M.E., et al. (2005) J. Biol. Chem., 280:4442-50] and live cell fluorescence and fluorescence resonance energy transfer (FRET) microscopy to dissect the molecular events in individual transport steps. A crucial step in the cycle involves sorting of both transport receptors, such as the carboxypeptidase Y (CPY)-receptor, Vps10p, and processing enzymes, such as Kex2 protease, at the TGN. These molecules are diverted from pathways to the early endosome and plasma membrane by transport to the late endosome/prevacuolar compartment (PVC), an event that depends on clathrin, the monomeric clathrin adaptor Gga, the dynamin homolog Vps1p, the Vps21p GTPase, the SM protein Vps45p and the PVC syntaxin Pep12p, but is independent of the AP-1 clathrin adaptor. Directed 2-hybrid and biochemical assays demonstrate that the VHS domain of Gga binds to sequences in both the Vps10p and Kex2p tails. Gga binds to a novel sequence in the Kex2p tail that includes an essential Ser residue (780). Binding is seen to a tail sequence containing the phosphomimetic substitution to Asp780, but not an Ala780 substitution. The Asp780 and Ala780 substitutions have opposite effects both on localization of Kex2p in vivo and on transport of Kex2p in the cell-free assay. We are testing the hypothesis that phosphorylation of Ser780 regulates the partitioning of Kex2p between Gga-dependent trafficking to the PVC and transport to the early endosome. The role of Arf GTPase in the Gga-dependent trafficking step is being studied using live-cell FRET stoichiometry microscopy measuring the interaction between C-terminal fusions of Arf1p to mCherry RFP and Gga2p to Venus YFP.

180/B123

Mitochondrial Protein-Import Mechanisms in Dinoflagellates.

J. C. Ackland, R. F. Waller; Botany, University of Melbourne, Melbourne, VIC, Australia

Dinoflagellate mitochondria have one of the most highly reduced genomes known, encoding only 3 proteins. All other genes essential for mitochondrial function have been re-located to the cells nucleus. In animals and fungi, the products of these genes are synthesised in the cytosol as precursors and targeted to the mitochondrion by an internal or cleavable N-terminal (presequence) targeting peptide. Translocases of the outer (TOM) and inner (TIM) mitochondrial membrane recognise these targeting signals and mediate protein import through two main sorting routes. Proteins containing N-terminal presequences are recognised by receptors Tom20 and Tom22, translocated through the general import pore (Tom40) and targeted to the matrix by the Tim23 complex. Alternatively, inner membrane carrier proteins that bear internal targeting sequences interact with Tom70 and Tom40, are guided through the intermembrane space by the Tim9-Tim10 complex and inserted into the inner membrane by the Tim22 complex. While the two sorting routes to mitochondria are conserved in animals and fungi, little is

known about the sorting routes in more diverse eukaryotes. We have examined the mechanisms of mitochondrial protein import in the dinoflagellate *Karlodinium micrum* to determine if multiple mitochondrial import pathways exist in dinoflagellates. Computational tools were used to predict chemical and structural properties of presequences, and protein targeting was tested using a heterologous yeast expression system. *K. micrum* mitochondrial presequences demonstrate similarities in amino acid usage and secondary structure compared to mitochondrial precursors of fungi, plants and animals. This conservation is sufficient for *K. micrum* presequence-bearing pre-proteins to be imported into the mitochondria of yeast, a distantly related eukaryote. Furthermore, we have provided evidence for internal targeting signals in *K. micrum* carrier proteins, which are similarly recognisable by yeast mitochondria. This study demonstrates how extensive the conservation of mitochondrial protein import mechanisms are across diverse eukaryotes.

181/B124

Altered Carbohydrate Fine Specificity of Galectin-3 Influences Biological Activity.

E. Salomonsson[1], M. C. Carlsson[1], R. Hendus-Altenburger[1], C. Öberg[2], R. Nilsson[1], V. Johansson[3], A. Carlsson[3], D. Delacour[4], R. Jacob[4], U. J. Nilsson[2], H. Leffler[1]; [1]MIG, Laboratory Medicine, Lund, Sweden, [2]Organic chemistry, Lund, Sweden, [3]Rheumatology and Inflammation Research, The Fagocyte Research Laboratory, Göteborg, Sweden, [4]Cell Biology and Cell Pathology, Marburg, Germany

Galectins, a family of small carbohydrate binding proteins, have been strongly implicated in inflammation and cancer. They are defined by a carbohydrate recognition domain (CRD) with a conserved sequence motif that forms a core binding site with affinity for b-galactosides. Most of the extracellular functions of galectin-3 require its carbohydrate binding activity conferred by the CRD, but its unique N-terminal part is also required for cross-linking of ligands and receptor activation. Galectin-3 also has intracellular effects, e.g. anti-apoptosis and in vesicular trafficking, but here the role of the carbohydrate binding activity is less clear. The carbohydrate fine specificity of galectin-3 has been thoroughly investigated, but its biological effects have not been studied much. We have therefore selectively fine tuned the specificity by point mutations in the CRD and studied the effect of this in biological systems: cell surface binding and neutrophil activation as examples of extracellular effects, and targeting of GFP-galectin-3 to vesicles as example of an intracellular effect. A clear correlation was found between fine specificity in solution, as measured with fluorescence anisotropy, and biological activity. The most striking effect was the total loss of affinity for the galectin-3 preferred ligand LacNAc when substituting Arg186 to Ser or Ile (R186S and R186I); these mutants were incompetent in binding to Jurkat cell surface and in activating neutrophils, and did not enter intracellular vesicles. Another mutant, K176L, had gained affinity for longer saccharides and lost affinity for sialylated saccharides. This moderate adjustment in carbohydrate fine specificity made the galectin a more potent neutrophil activator while cell surface binding declined. The R144S mutant had increased affinity for blood-group A-tetrasaccharide, a characteristic ligand of galectin-3, but decreased neutrophil activation. In conclusion, the biological functions of galectin-3 are not determined only by its galactoside binding ability per se, but also by its fine specificity for more complex saccharides. The mutants help to establish a link between particular aspects of the fine specificity with different biological functions.

182/B125

Screening of Recycling Endosomal Rab Small-GTPases with COS-1 Cells.

R. Misaki[1], D. Brown[1], M. Fukuda[2], J. Stow[1], T. Taguchi[1]; [1]Institute of Molecular Bioscience, University of Queensland, Brisbane, QLD, Australia, [2]Department of Developmental Biology and Neurosciences, Graduate School pf Life Sciences, Tohoku University, Sendai, Miyagi, Japan

Recent studies have revealed that recycling endosomes (REs) are organelles equipped with many functions. For example, REs are necessary for exocytic- and retrograde- transport pathways. However, how REs regulate this complex traffic remains mostly elusive. REs are often found in the perinuclear region, and located very close to the Golgi complex. This spatial restriction has hampered the detailed analysis of membrane transport pathways through the REs. We have recently found that COS-1 cells (green monkey kidney cells) possess a unique spatial distribution of endo- and exocytic organelles. REs are confined within the ring-shaped structure of the Golgi, whereas organelles for degradation (early endosomes, late endosomes, and lysosomes) are excluded. Rab proteins are key regulators of membrane traffic, and form the largest family of small GTPases. In this study, we screened recycling endosomal Rab proteins in COS-1 cells. Sixty-different GFP-fused mouse or human Rab proteins were individually expressed in COS-1 cells, and REs localization was validated with (a) colocalization of internalized transferrin and with (b) spatial distribution, i.e., being within the Golgi ring. Several Rabs identified as REs residents in COS-1 cells were further examined in polarized MDCK cells and RAW macrophages. This result will provide new insights into the function and compartmentalization of REs in various types of cells.

183/B126

Arabidopsis HSC70-1 and WPP-domain Proteins Prevent Aggregation and Promote Nuclear Envelope Targeting of Arabidopsis Tail-anchored Protein WIT1.

J. Brkljacic, Q. Zhao, I. Meier; Department of Plant Cellular and Molecular Biology, The Ohio State University, Columbus, OH

Tail-anchored (TA) proteins are a specialized class of diverse transmembrane proteins with different subcellular locations. They are characterized by a carboxyl-terminal hydrophobic transmembrane domain that dictates post-translational membrane integration. Molecular chaperones, namely Hsc70 and Hsp40, known for their role in folding and translocation of proteins across membranes, have recently been shown to be involved in targeting of several TA proteins in vitro. Arabidopsis WPP-domain proteins, WPP1 and WPP2, are plant-unique, nuclear envelope-associated proteins of unknown function. By tandem affinity purification coupled with mass spectrometry, we have identified two in vivo-interacting partners of WPP1 and WPP2 - an HSC70 chaperone and a plant-specific putative TA protein, WIT1 (WPP domain-interacting TA protein). WIT1 and GFP-WIT1 are targeted to the nuclear envelope in Arabidopsis thaliana. In contrast, GFP-WIT1 forms cytoplasmic aggregates when overexpressed transiently in Nicotiana benthamiana leaf epidermis cells. Coexpression of HSC70-1, WPP1 or WPP2 significantly reduces GFP-WIT1 aggregation and permits association of most GFP-WIT1 with the nuclear envelope. HSC70-1, WPP1 and WPP2 specifically interact with WIT1 in vivo and a ternary complex consisting of HSC70-1, WPP1 and WIT1 forms. A WPP1 mutant with reduced affinity for GFP-WIT1 fails to decrease its aggregation. While WPP-domain proteins act on a region of WIT1

containing the coiled-coil domain, HSC70-1 additionally acts on the C-terminal transmembrane domain. Taken together, we propose a novel role for HSC70-1 in targeting of a TA-protein in plants and describe the first in planta function for WPP-domain proteins.

184/B127

Regulation of Nutrient Permease Trafficking by Arrestin-like Yeast Proteins.

A. F. O'Donnell[1], R. G. Gardner[2], A. Apffel[3], M. S. Cyert[1]; [1]Biology Department, Stanford University, Stanford, CA, [2]Department of Pharmacology, University of Washington, Seattle, WA, [3]Agilent Technologies, Santa Clara, CA

Beta-arrestins are versatile proteins that regulate desensitization, endocytosis and intracellular trafficking of G-protein coupled receptors, and promote G-protein independent signaling. Though β-arrestin function is well understood, the related α-arrestin protein family has not been functionally defined in any organism. *S. cerevisiae* encodes 7 α-arrestin proteins. We show that two α-arrestins in yeast are functionally similar to β-arrestins and have named these Arrestin-Like Yeast proteins, or Aly1 and Aly2. Similar to β-arrestins, Aly1 and Aly2 co-localize and interact with clathrin and endocytic proteins, placing them in the appropriate location to regulate membrane-protein endocytosis or trafficking. Aly1 and Aly2 also interact with, and are ubiquitinated by, Rsp5. Similar to mammalian arrestins, this ubiquitination is essential for Aly function. Aly1 and Aly2 participate in nutrient signaling: over-expression of Aly1 or Aly2 stimulates yeast growth under conditions that mimic nitrogen-starvation, and deletion of either gene decreases growth under these conditions. To elucidate Aly1 and Aly2 function, we identified Aly-interacting proteins by mass spectrometry. Aly1 and Aly2 interact with the General amino acid permease, Gap1, which transports a wide range of nitrogen sources to assist in growth under nitrogen-poor conditions. Furthermore, Aly2 interacts with Npr1 (Nitrogen permease re-activator), a kinase required to stabilize Gap1 at the plasma membrane. Since Npr1 is required for Gap1 PM localization, cells lacking Npr1 are unable to import certain nitrogen sources and fail to grow under this nutrient limitation. Aly1 and Aly2 rescue growth of cells lacking Npr1 under these conditions, suggesting that Aly1 and Aly2 promote Gap1 activity in the absence of Npr1. Consistent with this idea, cells lacking Aly proteins display reduced Gap1 protein levels. We are currently testing the hypothesis that Aly1 and Aly2 promote growth in nitrogen-poor conditions by regulating Gap1 permease trafficking and/or activity, and are examining the impact of Npr1 on Aly function. These studies will establish a paradigm for investigating the function of other members of the α-arrestin family in eukaryotes.

Protein Targeting to the Cell Surface (185-195)

185/B128

Global Loss of Caveolae and Dyslipidemia in Cavin/PTRF Null Mice.

P. F. Pilch[1], L. Liu[1], K. Ravid[1], D. Brown[2], N. LeBrasseur[1], D. Yang[1], M. McKee[2], K. Albrecht[1]; [1]Boston University School of Medicine, Boston, MA, [2]Massachusetts General Hospital, Boston, MA

Caveolae are specialized invaginations of the plasma membrane that are found in numerous cell types, and they require caveolin protein expression for their formation. Although they have been implicated in a wide variety of cellular processes including signal transduction pathways, their function(s) remains enigmatic. We show by means of targeted gene disruption in mice that a distinct caveolae-associated protein, Cavin/PTRF, is an essential component of caveolae. Animals lacking Cavin have no morphologically detectable caveolae in any cell type examined and the expression of all three caveolin isoforms is reduced by >90% in these animals. Cavin knockout mice are viable and of normal weight, but have reduced adipose tissue mass, glucose intolerance and dyslipidemia. Insulin signaling is also diminished in the Cavin null mice but it is not completely clear if this is the cause of the metabolic phenotype. CD36, a lipoprotein receptor implicated in metabolic disorders and a resident protein of caveolae, is also reduced by ca. 90% in the knockout mice. Thus, our results underscore the multi-organ role of caveolae in metabolic regulation and provide a new paradigm for the study of caveolae formation, composition and function.

186/B129

The Role of Glycophorin A in the Trafficking of Anion Exchanger 1 to the Plasma Membrane.

A. J. Pang, R. Reithmeier; Biochemistry, University of Toronto, Toronto, ON, Canada

Anion exchanger 1 (AE1) is a membrane glycoprotein expressed in red blood cells (RBCs) where it mediates the electroneutral exchange of chloride and bicarbonate across the plasma membrane. Glycophorin A (GPA) is a sialoglycoprotein found in similar abundance to AE1 in RBCs. Previous studies have shown that these two proteins associate not only in the membrane, but also during biosynthesis with GPA facilitating the cell surface expression of AE1. The purpose of this study is to investigate the role of GPA in the trafficking of AE1 from the ER to the plasma membrane in K562 cells, a human erythroleukemic cell line that endogenously expresses GPA. GPA in K562 cells was shown to be N- and O-glycosylated and migrated as a dimer on SDS polyacrylamide gels, similar to erythroid GPA. GPA was found at the cell surface and intracellularly, co-localizing with ER and Golgi markers. We were able to successfully express wild-type HA-tagged AE1 in K562 cells and these cells were used to investigate GPA-AE1 interactions. Wild-type AE1 expressed in K562 cells was processed to a complex sugar form and co-localized with GPA at the cell surface and intracellularly. The GPA-AE1 complex was confirmed by co-immunoprecipitation studies. The effect of GPA knock down by shRNA on the cell surface expression of AE1 was studied.

187/B130

Mutational Analysis of the Nucleotide Receptor P2X7 Reveals Key Residues Linked to the Localization, Trafficking and Glycosylation of This Major Immunoregulatory Protein.

L. Lenertz, Z. Wang, P. Bertics; Biomolecular Chemistry, University of Wisconsin-Madison, Madison, WI

In response to injury or infection, cells can release millimolar levels of ATP. This extracellular ATP can bind to the nucleotide receptor P2X7 and can enhance the immune response through several mechanisms, including the promotion of Interleukin-1b release. P2X7 functions as a cation channel but it also stimulates the formation of a non-specific pore after prolonged activation, allowing for molecules of up to 900 Da to enter the cell. Several lines of evidence suggest that P2X7 trafficking may be critical for modulating cell responsiveness to ATP yet the

molecular mechanisms of this important process are unknown. Because oligomeric plasma membrane proteins are generally synthesized and retained in the ER until the protein subunits are properly assembled, we first evaluated P2X7 localization in the ER using immunofluorescence. We observed that both over-expressed and endogenous P2X7 are predominantly intracellular and co-localize with the ER marker protein disulfide isomerase. Next, based on sequence comparison to other plasma membrane receptors, we tested the hypothesis that P2X7 contains an arginine-based ER retention signal in its distal C-terminus. In these studies, we found that mutation of R576 and R578 to alanines greatly attenuates receptor activity, as assessed by agonist-stimulated fluorescent dye uptake, thereby suggesting that mutating these residues causes P2X7 to exit the ER prematurely before it is properly assembled. In addition, we have begun testing the idea that P2X7 trafficking and activity are regulated by N-linked glycosylation. Using mutational analysis and endoglycosidase H assays, we have established that P2X7 is N-linked glycosylated on N187, N202, N213, N241 and N284. These investigations represent the first mapping of the glycosylation sites on P2X7, and we are now assessing whether mutation of these amino acids reduces P2X7 cell surface expression and activity. Initial results suggest that mutation of N202 and N284 to alanine does not affect P2X7 function. A greater understanding of P2X7 trafficking will aid in the elucidation of the process by which this major immunomodulatory protein and potential therapeutic target contributes to a spectrum of inflammatory diseases.

188/B131

Retromer Function in the Plasma Membrane Recycling Path of Beta 2 Adrenergic Receptor.

P. Temkin, M. von Zastrow; UCSF, San Francisco, CA

Classically, retromer function has been associated with trafficking of membrane cargo from endosomes to the Golgi apparatus. Using RNA interference in cultured human embryonic kidney (293) cells, I have obtained evidence that the retromer also functions in recycling the Beta 2 Adrenergic Receptor (B2AR) from endosomes to the plasma membrane. Furthermore, using confocal imaging of living cells, I have observed that the retromer marks a distinct subset of endosomal recycling tubules through which the B2AR traffics. This suggests a novel function of the retromer in sequence directed recycling and begs reevaluation of current dogma for retromer utility.

189/B132

Effects of Polycystin-2 on Polycystin-1 Localization and Cleavage.

H. C. Chapin[1], C. Bertuccio[2], M. Caplan[2]; [1]Cell Biology, Yale University, New Haven, CT, [2]Cellular and Molecular Physiology, Yale University, New Haven, CT

Polycystin-1 (PC1) is a large, membrane-bound protein that is localized to the plasma membranes and cilia of epithelial cells where it may play a role as a mechano- or chemosensory receptor. PC1 interacts with the calcium channel Polycystin-2 (PC2), and this interaction can affect the localization of both proteins in cell culture as well as the channel properties of PC2. It remains unclear, however, which functional and structural attributes of PC2 allow it to modify PC1 localization. To address this question we use immunofluorescence to show that co-expression of PC2 is required for PC1 to reach the cell surface. Expressing a channel-inactive mutant of PC2 does not support robust surface localization of PC1, while a truncation mutant of PC2 that cannot stably interact with PC1 does allow PC1 to exit the ER and reach the cell surface. This demonstrates that a functional PC2 channel is required for PC1 to reach the cell surface in cell culture; additionally, this shift of PC1 to the surface is independent of PC2 localization and does not require a stable interaction of PC1 with PC2. Polycystin-1, for its part, can move to the cell surface only if it can be autocatalytically cleaved at its G-protein coupled receptor proteolytic site (GPS). Though co-expression of wild-type PC2 greatly enhances this cleavage, there is no significant increase in cleavage associated with the co-expression of the truncation mutant of PC2 that supports PC1 surface delivery, indicating that PC2's stimulatory effect on cleavage is independent of its capacity to support PC1 surface localization. In summary, our data indicate that PC2 channel activity is necessary to bring PC1 to the cell surface and that GPS cleavage of PC1 is necessary but not sufficient for surface localization.

190/B133

Arf6 and SCAMP2 Regulate the Plasma Membrane Targeting of the Brain-enriched Na+/H+ Exchanger NHE5.

G. Diering[1], J. Church[1], M. Numata[2]; [1]Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada, [2]Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada

Abstract NHE5 is a brain-enriched Na+/H+ exchanger that plays a pivotal role in pH homeostasis by exchanging extracellular Na+ for intracellular H+. NHE5 dynamically shuttles between the plasma membrane and recycling endosomes, serving as a mechanism that acutely controls the local pH environment. In the current study we show that Secretory Carrier Membrane Proteins (SCAMPs), a group of tetraspanning integral membrane proteins that reside in multiple secretory and endocytic organelles, bind to NHE5 and coloclize predominantly in the recycling endosomes. In vitro protein-protein interaction assays revealed that NHE5 directly binds to the N- and C-terminal cytosolic extensions of SCAMP2. Heterologous expression of SCAMP2 but not SCAMP5 increased cell surface abundance as well as transporter activity of NHE5 across the plasma membrane. Expression of a deletion mutant lacking the SCAMP2-specific N-terminal cytosolic domain, and a mini-gene encoding the N-terminal extension reduced the transporter activity. The SCAMP-NHE5 complex co-localized with both Arf6 and Rab11. We further show that while both Arf6 and Rab11 positively regulate NHE5's cell-surface targeting and NHE5-activity across the plasma membrane, SCAMP2-mediated surface targeting of NHE5 was reversed by dominant-negative Arf6 but not by dominant-negative Rab11. Together, these results suggest that SCAMP2 regulates NHE5 transit through recycling endosomes and promotes its surface targeting in an Arf6-dependant manner.

191/B134

Synaptic Palmitoylation of PSD-95 Mediates AMPA Receptor Homeostasis.

M. Fukata[1,2], J. Noritake[1], T. Iwanaga[1], R. Tsutsumi[1], Y. Fukata[1,2]; [1]Division of Membrane Physiology, Department of Cell Physiology, National Institute for Physiological Sciences, Okazaki, Japan, [2]PRESTO, Japan Science and Technology Agency, Chiyoda, Japan

Homeostatic regulation of synaptic AMPA receptors participates in the neuronal plasticity that underlies learning and memory. The palmitoylated postsynaptic scaffolding protein, PSD-95, determines the density of synaptic AMPA receptors and may regulate this process.

Here, we report that blocking synaptic activity rapidly augments PSD-95 palmitoylation and recruits synaptic AMPA receptors. A dendritically localized palmitoyl-transferase DHHC2, but not the Golgi-resident DHHC3, mediates this activity-sensitive PSD-95 palmitoylation. Upon activity blockade, DHHC2 translocates to the postsynaptic density to mediate this effect. These data demonstrate that dynamic recruitment of protein palmitoylation machinery plays a central role in regulating synaptic homeostasis.

192/B135

Regulation of Gap1p Trafficking Requires Direct Sensing of Amino Acids.

N. Cain, C. Kaiser; MIT, Cambridge, MA

The general amino acid permease Gap1p of *Saccharomyces cerevisiae* is subject to regulated trafficking in response to amino acids. Gap1p is transported to the plasma membrane in the absence of amino acids but is sorted to the vacuole when amino acids are abundant, thus preventing the uptake of toxic quantities of amino acids from the extracellular medium. Under conditions of high extracellular amino acids most of the intracellular Gap1p en route to the vacuole is located in the membrane of the multivesicular endosome (MVE). If amino acid levels fall Gap1p can be mobilized from the MVE to the plasma membrane. The mechanisms by which amino acids are sensed and intracellular sorting of Gap1p is regulated are poorly understood. We have found a substrate specificity mutation of Gap1p, A297V, which blocks transport of positively charged amino acids such as lysine and arginine, while preserving the ability to transport other amino acids, including glycine, phenylalanine, and threonine. We have designed experiments to test the sorting regulation of $Gap1p^{A297V}$ and find that intracellular sorting of this mutant has specifically lost the ability to respond to positively charged amino acids. These results indicate that Gap1 functions as its own amino acid sensor to direct sorting to the plasma membrane or retention in internal compartments.

193/B136

Direct Interaction between a Myosin V Motor and the Rab GTPases Ypt31/32 Is Required for Polarized Secretion.

Z. Lipatova[1], A. A. Tokarev[1], Y. Jin[2], L. Weisman[2], J. Mulholland[3], N. Segev[1]; [1]Biological Sciences, University of Illinois, Chicago, IL, [2]University of Michigan, Ann Harbor, MI, [3]Stanford University, Stanford, CA

Rab GTPases recruit myosin motors to endocytic compartments, which in turn are required for their motility. However, no Ypt/Rab GTPase has been shown to regulate the motility of exocytic compartments. In yeast, the Ypt31/32 functional pair is required for the formation of trans-Golgi vesicles. The myosin V motor, Myo2, attaches to these vesicles through its globular-tail domain (GTD) and mediates their polarized delivery to sites of cell growth. Here, we identify Myo2 as an effector of Ypt31/32 and show that the Ypt31/32-Myo2 interaction is required for polarized secretion. Using the yeast-two hybrid system and co-precipitation of recombinant proteins, we show that Ypt31/32 in their GTP-bound form interact directly with Myo2-GTD. The physiological relevance of this interaction is shown by co-localization of the proteins, genetic interactions between their genes, and rescue of the lethality caused by a mutation in the Ypt31/32-binding site of Myo2-GTD through fusion with Ypt32. Furthermore, microscopic analyses show a defective Myo2 intra-cellular localization in ypt31/32ts and in Ypt31/32-interaction-deficient myo2 mutant cells, as well as accumulation of un-polarized secretory vesicles in the latter mutant cells. Together, these results indicate that Ypt31/32 play roles in both the formation of trans-Golgi vesicles and their subsequent Myo2-dependent motility.

194/B137

Regulated Endocytosis of Prm1 in Yeast Mating Pairs.

V. Olmo, E. Grote; Biochemistry and Molecular Biology, Johns Hopkins University Bloomberg School of Public Health, Baltimore, MD

Cell-cell fusion is essential for skeletal muscle formation, bone remodeling, placenta formation, and the union of sperm and egg to create a zygote. Mating in *Sacchromyces cerevisiae* provides a genetically accessible model system to study cell-cell fusion. The pheromone regulated membrane glycoprotein Prm1 acts at the plasma membrane fusion step of mating. In mating pairs, GFP-Prm1 is clearly visible at its expected site of action- the plasma membrane at the site of cell-cell contact. In contrast, Prm1 is difficult to detect on the plasma membrane when it is expressed in non-mating cells. Instead, it colocalizes with two endocytic markers, Ste6-GFP and GFP-FYVE, and has a half-life of approximately 15 minutes. Mutations in a putative endocytosis signal, DPFxD, caused retention at the plasma membrane. These results suggest that in the absence of a mating signal, Prm1 is delivered to the plasma membrane but is then immediately internalized and targeted to vacuoles. Mutation of the DPFxD signal did not affect mating activity. Our model is that Prm1 endocytosis is inhibited by cell-cell contact, thereby allowing Prm1 to accumulate on the plasma membrane and participate in plasma membrane fusion.

195/B138

Posttranslational Modification of Chlamydomonas HAP2 Is Essential for Protein Localization and Gamete Fusion in Chlamydomonas.

Y. Liu, W. J. Snell; Cell Biology, University of Texas Southwestern Medical Center at Dallas, dallas, TX

Previously, mutants of the HAP2 gene in Arabidopsis were shown to be male sterile (Johnson et al. 2004 Genetics 168:971). The HAP2 gene product is present on Arabidopsis sperm (Mori et al. 2006 Nat Cell Biol 8:64) and also essential for pollen tube guidance (von Besser et al. 2006 Develop 133:4761). The cellular function of HAP2 was unknown. In recent studies in the unicellular green alga Chlamydomonas and the malaria organism Plasmodium, we showed that HAP2 is essential at the gamete membrane fusion step that follows species-specific gamete membrane adhesion. HA-tagged HAP2 in Chlamydomonas mt- gametes migrates as two isoforms in SDS-PAGE, and only the upper form is sensitive to trypsin, suggesting that it is uniquely exposed at the cell surface (Liu et al. 2008 Genes&Dev. 22:1051). Here, we report that HAP2 posttranslational modification is required for localization to the apical membrane patch specialized for fusion. RT-PCR detects only a single, full-length HAP2 transcript indicating that the two isoforms are not a result of alternative splicing. HAP2 driven by a constitutive promoter is expressed only as the lower form in vegetative cells, indicating that the modification is gamete-specific. HAP2 constructs modified in any of several regions directed expression of only the lower form and failed to rescue fusion in a HAP2 mutant. We find no evidence for phosphorylation or sumolylation of HAP2. Furthermore, both isoforms of HAP2 were glycosylated, but only the lower

isoform was PNGaseF-sensitive. Characterizing the modification should uncover protein-protein interaction domains. Identification of the HAP2 posttranslational modification by mass spectrometry is underway. Supported by NIH GM56778 to WJS.

Membrane Receptors (196-220)

196/B139

NaKTide, Na/K-ATPase-derived Peptide Src Inhibitor, Antagonizes Ouabain-activated Signal Transduction in Cultured Cells.

Z. Li, T. Cai, L. Liu, J. Tian, J. X. Xie, X. Zhao, Z. Xie; Physiology and Pharmacology, University of Toledo Health Science Campus, Toledo, OH

Many studies have demonstrated nanomolar concentration of ouabain activates Src through Na/K-ATPase/Src receptor complex, and then consequently induce activation of several signaling pathways, including activation of MAPK pathway, mitochondrial release of reactive oxygen species (ROS), and transient release of intracellular Ca2+. The formation of Na/K-ATPase/Src complex involves the interaction between the third cytosolic domain (CD3) of α1 and kinase domain of Src. This interaction functionally keeps Src in inactive state, while ouabain binding will release the kinase domain and lead to Src activation. However, it is still unknown how the CD3 inhibits Src. Here, we further identified the binding motifs within the CD3. First, GST pulldown assay showed a direct interaction between the N-terminus (ND1) of N domain (nucleotide-binding domain) of α1 and Src. FRET analysis indicated ND1 was able to associate with Na/K-ATPase/Src complex in LLC-PK1 cells. Functionally, ND1 significantly inhibited Src activity in test tubes as well as LLC-PK1 cells. Second, a 20-residue peptide, denoted as NaKTide, encompassing amino acid residues from ND1, was capable to inhibit Src in a dose-dependent manner. Accordingly, cell-permeable NaKTide consisting of NaKTide coupled to GRKKRRQRRRPPQ significantly inhibited Src with IC50 around 4 nM, while no inhibitory effect on PKC activity up to 10 μM. Consistently, permeable NaKTide inhibited basal Src as well as ERK activity in PY-17 cells where these kinases activities were elevated specifically due to knockdown of Na/K-ATPase. Finally, preincubation with permeable NaKTide abrogated ouabain-induced activation of Src and then blocked MAPK signaling pathway in both LLC-PK1 and neonatal cardiac myocytes. These new findings indicate that NaKTide may act as a potential ouabain antagonist. Moreover, NaKTide makes it possible for us to further address the function role of digitalis-induced signaling through Na/K-ATPase.

197/B140

Long-term Regulation of Na,K-ATPase in Opossum Kidney Cells by Ouabain.

E. Silva, P. Soares-da-Silva; Faculty of Medicine, Institute of Pharmacology and Therapeutics, Porto, Portugal

Ouabain is a classical Na,K-ATPase inhibitor, but also activates several signaling cascades including the phosphoinositide 3 kinase (PI-3K) and the MEK pathway, which leads to modifications in the gene and protein expression. The aim of this work was to study the long-term regulation of Na,K-ATPase by ouabain activated signaling in opossum kidney (OK) cells, at passages 40 and 80 in culture. For long-term regulation studies (OK) cells (acquired from ATCC with 36 passages) were incubated with ouabain in the presence or absence of specific PI-3K and MAPK inhibitors during 4 days. Na,K-ATPase activity was evaluated by electrophysiological methods and α1-subunit Na,K-ATPase expression was evaluated by western blotting. Na,K-ATPase activity and expression increased with the number of OK cells passages. When incubated with ouabain (100 nM) during 4 days Na,K-ATPase activity and α1-subunit Na,K-ATPase expression was not altered in cells with 40 passages. However, in OK cells with 80 passages incubation with ouabain (100 nM) induced an increase in Na,K-ATPase activity from 16.14±2.68 μA/cm2 to 26.51±3.78 μA/cm2 and a 2.12±0.18 fold increase in relative α1-subunit Na,K-ATPase expression. Inhibition of PI-3K (wortmannin - 10 nM) or MEK (PD 098059 - 10 μM) prevented ouabain-induced increase in α1-subunit Na,K-ATPase expression. Incubation with the p38 inhibitor SB 203580 (10 μM) failed to revert the ouabain-induced increase in α1-subunit Na,K-ATPase expression. Ouabain-induced increase in Na,K-ATPase activity was prevented when OK cells were treated with PI-3K, MEK or p38 inhibitors. It is concluded that mechanisms set into motion during long-term ouabain-induced increases in Na,K-ATPase expression and activity include the activation of PI-3K and the MEK pathways.

198/B141

Regulation of Intracellular Cholesterol Distribution by the Na/K-ATPase.

Y. Chen[1], H. Wang[1], T. Cai[1], Z. Li[1], E. Loreaux[2], J. B. Lingrel[2], Z. Xie[1]; [1]Physiology and Pharmacology, University of Toledo, Toledo, OH, [2]Molecular Genetics, Biochemistry and Microbiology, University of Cincinnati, Cincinnati, OH

The Na/K-ATPase was discovered as an energy transducing ion pump. Recent studies have ascribed many non-pumping functions to the Na/K-ATPase. We showed here that graded knockdown of the cellular Na/K-ATPase produced a parallel decrease in cholesterol in the caveolar fractions of LLC-PK1 cell lysates. This observation was further substantiated by imaging analysis, showing redistribution of cholesterol from the plasma membrane to intracellular compartments in the knockdown cells. Moreover, this regulation was confirmed in the Na/K-ATPase α1+/- mouse liver. Functionally, the Na/K-ATPase knockdown decreased cholesterol content in the endoplasmic reticulum (ER), resulting in an activation of the sterol regulatory element binding protein 2 (SREBP2) and a subsequent increase in the expression of HMG-CoA reductase and the low density lipoprotein (LDL) receptor in the liver. Consistently, the knockdown caused a modest increase in hepatic cholesterol as well as a reduction in plasma cholesterol. Furthermore, we presented evidences that Na/K-ATPase α1 subunit directly interacted with cholesterol revealed by fluorescence energy transfer (FRET) analysis, which may represent an important sensing mechanism by which the cells regulate ER cholesterol and then the SREBP2 pathway.

199/B142

ATP Modulates Cl--channels in GFSHR-17 Granulosa Cells, the In Vitro Model of Granulosa Cells in Maturing Follicle.

W. Bintig[1], J. Baumgart[2], W. J. Walter[3], A. Heisterkamp[2], H. Lubatschowski[2], A. Ngezahayo[1]; [1]Inst. f. Biophysik, Leibniz Universität Hannover, Hannover, Germany, [2]Laserzentrum Hannover e.V., Hannover, Germany, [3]Molecular and Cell Physiology, Hannover Medical School, Hannover, Germany

Purinergic signalling in GFSHR-17 granulosa cells was characterised by intracellular Ca^{2+}-imaging and perforated patch-clamp technique. A resting intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) of 100 nM and a membrane potential (U_m) of -45 mV were observed. The U_m is consistent with a high permeability to K^+ and Cl^- and to a high intracellular Cl^- concentration. Repeated ATP applications for 5-15 s every 3 min resulted in Ca^{2+}-release and hyperpolarisation to -70 mV. Increasing the time of ATP presence or reducing the interval between consecutive applications induced desensitisation. Phospholipase C inhibitor (U73122) or IP_3 receptor blocker (2-APB) suppressed ATP-related responses. Further biochemical and pharmacological experiments revealed that the responses to ATP were related to P2Y2 and P2Y4 receptor subtypes, and that Ca^{2+}-increase was a prerequisite for hyperpolarisation. The hyperpolarisation could be related to a K^+-channel activation or to an inhibition of Cl^- channels. Prediction of U_m using the Goldmann-Hodgkin equation when ATP was applied under different conditions such as increased K^+-concentration or substitution of Cl^- in external solution could not distinguish whether K^+ channels were activated or Cl^- permeability was inhibited. However Apamin, Iberiotoxin, clotrimazole and even TEA did not affect the ATP-induced responses. Inhibitors of Cl^- channels induced hyperpolarisation and suppressed further ATP-related hyperpolarisation which was predicted by the Goldmann-Hodgkin equation if the ATP were activating K^+ channels. We propose that P2Y2 and P2Y4 receptors in GFSHR-17 granulosa cells modulate the Cl^- permeability and the membrane potential by regulating intracellular Ca^{2+}-release.

200/B143

Use of Photoaffinity Labeling through Peptide Residues 28 and 31 and Molecular Modeling to Elucidate the Molecular Basis of Cholecystokinin Docking to Its Receptor.

M. Dong[1], P. C. Lam[2], D. I. Pinon[1], R. Abagyan[2], L. J. Miller[1]; [1]Mayo Clinic, Scottsdale, AZ, [2]Scripps Research Institute and Molsoft LLC, La Jolla, CA

The Type A cholecystokinin receptor is a member of Family A G protein-coupled receptors that include many important drug targets. Despite recent advances in structural determination of a few members of this family, understanding of the mechanisms of peptide ligand binding and activation of these receptors remains limited. Photoaffinity labeling is a powerful approach to establish spatial approximations between photolabile residues within a ligand and its receptor. In this work, we have utilized two cholecystokinin analogues with photolabile benzoyl-benzoyl lysine residues situated in positions 28 or 31 within its pharmacophoric domain. Both analogues were fully efficacious agonists and bound the cholecystokinin receptor with high affinity. They covalently labeled the receptor efficiently and saturably. The domains of labeling were identified by purification and proteolytic peptide mapping of labeled wild-type and mutant cholecystokinin receptors. The specific sites of receptor labeling were identified by radiochemical sequencing of purified cyanogen bromide fragments of these receptor constructs. The position 28 probe labeled second extracellular loop residue Leu^{199}, while the position 31 probe labeled first extracellular loop residue Phe^{107}. Along with five additional spatial approximation constraints coming from previous photoaffinity labeling studies and twelve distance restraints coming from fluorescence resonance energy transfer studies, these were built into a new β2-adrenergic receptor-based homology model of the cholecystokinin receptor. The resultant agonist ligand-occupied receptor model fully accommodates all existing experimental data and is felt to represent one of the best refined models of a peptide hormone receptor in this important family.

201/B144

Determination of the Molecular Basis of Glucagon-like Peptide 1 Docking to Its Receptor Using Photoaffinity Labeling through Peptide Residues 12 and 35.

Q. Chen, D. I. Pinon, L. J. Miller, M. Dong; Mayo Clinic, Scottsdale, AZ

The glucagon-like peptide 1 (GLP1) receptor is a member of Family B G protein-coupled receptors and is an important drug target for type 2 diabetes. The natural agonist ligand, GLP1, has incretin-like actions that hold promise for the management of this disease. Despite recent solution of the structures of the amino-terminal domains of the GLP1 receptor and several close family members, our understanding of the molecular basis of GLP1 binding to its receptor is still limited. Here, we used photoaffinity labeling to explore GLP1 docking to its intact receptor. We developed and characterized two high-affinity, full-agonist photolabile probes having sites for covalent attachment in position 12 and 35. Both labeled the receptor specifically and saturably, with this inhibited by GLP1 in a concentration-dependent manner. The photoaffinity labeled receptor migrated at approximate 66 kD and shifted to 42 kD after deglycosylation. The labeled receptor was then purified and submitted to chemical and proteinase cleavages. This identified the juxtamembrane region of the amino-terminal domain of the GLP1 receptor (between Ser^{135} and Arg^{170}) as the region of labeling for the position 12 probe. This was further localized to Leu^{144}-Arg^{170} by cleaving two GLP1 receptor mutants, S136M and F143M. Further, radiochemical sequencing of these mutant receptors identified receptor Tyr^{145}, a residue adjacent to the first transmembrane segment, as the specific site of labeling by the position 12 probe. Similarly, the region of labeling for the position 35 probe was localized to the segment Asp^{114}-Lys^{130} within the amino-terminal domain of the receptor. Receptor mutants are being made to identify the specific site of labeling by radiochemical sequencing. These data are consistent with the antagonist-occupied crystal structure of the GLP1 receptor amino terminus. As we expand the number of such experimental constraints, we should be able to meaningfully model the agonist-bound GLP1 receptor, adding to our insights into the molecular basis of ligand binding to this important receptor.

202/B145

Interaction of Novel Ibogaine Analogs with the Human α3β4 Nicotinic Receptor.

B. R. Obrock[1], H. Arias[2], D. Feuerbach[3]; [1]AZCOM, Midwestern University, Glendale, AZ, [2]Department of Pharmacy, Midwestern University, Glendale, AZ, [3]Neuroscience, Novartis Institutes for BioMedical Research, Basel, Switzerland

This work is an attempt to characterize the binding site and the inhibitory activity of ibogaine analogs on the human α3β4 nicotinic acetylcholine receptor (hα3β4 AChR). In this regard, we used [3H]ibogaine equilibrium binding and Scatchard-plots, [3H]ibogaine and [3H]epibatidine competition binding, and ibogaine-induced inhibition of Ca2+ influx approaches. The results indicate that: (1) there is one high-affinity binding site for [3H]ibogaine, (2) ibogaine inhibits the hα3β4 with higher potency than that for the α1β2γδ AChR, (3) [3H]ibogaine competition experiments indicate that ibogaine and 18-MAC, among ibogaine analogs, and imipramine and dextromethorphan, among other known noncompetitive antagonists, have the highest affinities for the hα3β4 ion channel in the resting state, (4) ibogaine analogs

are potent inhibitors of the hα3β4 AChR, and (5) [3H]epibatidine competition experiments indicate that ibogaine analogs interact with the agonist sites with very low affinities. Taken together, our results indicate that ibogaine analogs inhibit the hα3β4 AChR by interacting preferably with the epibatidine-activated ion channel.

203/B146

HSP90beta Regulates Rapsyn Turnover and Subsequent Acetylcholine Receptor Cluster Formation and Maintenance.

S. Luo, B. Zhang, X. Dong, Y. Tao, A. Ting, Z. Zhou, J. Meixiong, J. Luo, F. Chiu, W. Xiong, L. Mei; IMMAG, Medical College of Georgia, Augusta, GA

Rapsyn, an acetylcholine receptor (AChR)-interacting protein, is essential for synapse formation at the neuromuscular junction (NMJ). Like many synaptic proteins, rapsyn turns over rapidly at synapses. However, little is known about molecular mechanisms that govern rapsyn stability. Using a differential mass-spectrometry approach, we identified heat-shock protein 90beta (HSP90beta) as a component in surface AChR clusters. The HSP90beta-AChR interaction required rapsyn and was stimulated by agrin. Inhibition of HSP90beta activity or expression, or disruption of its interaction with rapsyn attenuated agrin-induced formation of AChR clusters in vitro and impaired the development and maintenance of the NMJ in vivo. Finally, we showed that HSP90beta was necessary for rapsyn stabilization and regulates its proteasome-dependent degradation. Together, these results indicate a role of HSP90beta in NMJ development by regulating rapsyn turnover and subsequent AChR cluster formation and maintenance.

204/B147

Expression of TRPV4 in the Stimulated Rat Oral Mucous Membrane -Nociception of Lingual Conical Papillae-.

M. Nakatsuka[1], S. Jue[2], S. Kumabe[1], K. Takama[1], J. Shin[2], Y. Iwai[1]; [1]Osaka Dental University, Hirakata City, Japan, [2]Kyung-Hee University, Seoul, South Korea

Transient receptor potential superfamily contains receptors activated by thermal and osmotic pressure stimuli. We studied the effect of TRPV4 expression on neuronal activation in the descending modulatory system in response to noxiception of the oral cavity. The intermolar region of dorsal lingual eminence of rats (250g, male; n= 6) was stimulated with filter paper discs soaked with 10μm of either normal saline (0% formalin; control) or 5% formalin (experimental) for 5 minutes. The tongues and brain stems were dissected, frozen-sectioned, and then the specimens were histo-immunologically stained for examination of TRPV4, pERK and 5HT immuno-reactive cells in specific regions. Histological findings of the experimental group demonstrated significant an increase of TRPV4 activity in particular in stratum basale and stratum granulosum of giant conical foliate lingual papillae (t test, $p<0.01$). 5HT immuno-reactive cells were significantly found in lamina propria ($p<0.01$), but not in the epithelium of lingual mucosa of the papillae. In the brain stem, pERK positive cells were significantly increased in the Sp5C ($p<0.05$) and Md ($p<0.01$), and TRPV4-positive cells were significantly found ($p<0.05$) showing a similar distribution with pERK-positive cells at the peripheral layer of Sp5C. On the other hand, a significant increase of 5HT activity was observed with in NTS ($p<0.05$), LRt ($p<0.05$), RPa ($p<0.01$), ROb ($p<0.05$) and RMg ($p<0.01$); no significant changes of 5HT activity was found in PAG and NRD. Our study provides that TRPV4 receptors in the oral mucosa are nociceptors of neurogenic inflammation and peripheral hyperalgesia, and pERK expression in Sp5C is closely related with central hyperalgesia (algesthesia?) of the nociception. Noxious stimulation on dorsal lingual mucosa elicits pERK-positive cells, which evoke TRPV4 in the Sp5C to amplify pain sensation. Furthermore, the study indicates that ERK cells of the central 5HT nervous system are activated to accelerate 5HT release for neuronal modulation of the descending pain modulatory system in response to noxiception.

205/B148

Intracellular Ca2+ Signaling and Volume Changes in an Immortalized Submandibular Salivary Gland Cell Line from the Rat.

M. H. Aure, H. K. Galtung; Department of Oral Biology, University of Oslo, Oslo, Norway

We wished to document the intracellular Ca^{2+} response in a SV40 immortalized rat submandibular cell line to adequate neurotransmitters and to investigate whether neurotransmitter stimuli resulted in volume changes. The cell line, a gift from D Quissell, U of Colorado, was grown under standard cell culture conditions. At the time of experiments, cells were incubated with fura-2-AM (Molecular Probes, USA) following established routines. The cells were studied in a flow through chamber with an inverted microscope. As a measure of the intracellular Ca^{2+} concentration, fura-2 was excited at 510 nm and the ratio of emitted light at 340 and 380 nm was recorded using MetaFluor 7.5.4.0 software (Universal Imaging, USA). After a few minutes in an isosmotic solution (300 mOsm, pH 7.4), the cells were exposed for 15 min to the neurotransmitters carbamoylcholine chloride (10^{-4} M) or ATP (10^{-4} M) and the changes in intracellular Ca^{2+} was measured. As a measure of cell size, changes in cell surface were measured using the Metamorph 7.5.4.0 software (Universal Imaging, USA). Control experiments were performed for both Ca^{2+} and volume measurements by exposing cells solely to isosmotic solution without neurotransmitters. Exposure to both neurotransmitters induced a transient increase in the intracellular Ca^{2+} level in all cells. The largest response was to carbamoylcholine chloride which exhibited an average Ca2+ increase of 377 ± 23% at 11 sec after neurotransmitter stimuli. Addition of the M3 receptor antagonist atropine (1μM) blocked this Ca^{2+} signal. When exposed to ATP, the intracellular Ca^{2+} increased by 237 ± 28% on average 9 sec after stimuli. We could not detect any volume changes in the cells as a response to neurotransmitter stimuli. There was, however, observed a transient blebbing in majority of the cells. In conclusion, in this cell line there was a strong intracellular Ca^{2+}-increase in response to both carbamoylcholine chloride and ATP. Thus, this cell line has retained a normal Ca^{2+}-response to transmitters of the autonomic nervous system. No volume changes could be detected during transmitter activation.

206/B149

Expressions of Protease-activated Receptors (PARs) in Retinal Pigment Epithelium (RPE) Cells.

M. Kim, J. Baik, I. Kong; Department of Physiology, Yonsei Univ. Wonju College of Medicine, Wonju, South Korea

Protease-activated receptors (PARs) are G-protein coupled receptors respond to extracellular protease. The retinal pigment epithelium (RPE) cell is a monolayer of cells lying between the retinal photoreceptors and the choroidal blood supply. RPE cells respond to oxidative stress, inflammation, and retinal degeneration. Until now, the expression profile of PARs is still unknown. In this study we identified the role of

PARs in RPE cells, such as HRPE and ARPE cell, using fluorescence Ca2+ imaging technique and real-time PCR. Thrombin and trypsin, PAR receptor agonists, increase the [Ca2+]i in a concentration-dependent manner (EC50=3.4±0.08 nM, EC50=25.4±0.13 nM, respectively). Selective peptide agonist of PAR1 (TFLLR-NH2) and PAR2 (SLIGRL-NH2) increase the [Ca2+]i. PAR agonists increase the [Ca2+]i independent of extracellular Ca2+ levels. Furthermore, U73122 (2 μM), a PLC inhibitor, completely blocked the Ca2+ increment by activation of PAR receptors. The inositol 1,4,5-trisphosphate receptors (IP3Rs 1~3) are expressed in RPE cells. All subtype of PAR receptors are identified and PAR1, PAR3 dominantly expressed. PAR receptor agonists exposure significantly increased mRNA expression of the proinflammatory cytokine interleukin-6 (IL-6), tumor necrosis factor-α (TNFα). Taken together, we suggest that PAR receptors in RPE cells may contribute to the pathogenesis of inflammatory disease.

207/B150

Role of Beta Arrestins In VEGF Signaling and Endothelial Permeability Response.

P. Kumar, M. H. Wu, S. Y. Yuan; Department of Surgery, University of California Davis Medical Center, Sacramento, CA

Beta arrestins, originally identified based on their role in arresting the G-protein coupled receptor signal transduction, have now been implicated in various molecular reactions, including the termination of signaling response to receptor tyrosine kinases. Two different isoforms, beta 1 and 2 arrestins, have been identified in different cells and tissues. Ever since their discovery they've been traditionally assumed to work in a redundant and interchangeable fashion. However recent evidence points towards more specialized roles of individual isoforms in not only the termination of signaling but also the specificity of signaling through different receptors. Here we tested the potential roles of different beta-arrestin isoforms in the endothelial permeability response to VEGFR2, a tyrosine kinase receptor that plays a major role in VEGF signaling. Our experiments using live cell imaging, electrical cell impedance sensor, and western blotting showed that the presence of one or both beta arrestins not only affected VEGFR2-mediated Ca+2 response and its downstream kinase activation, but also led to an overall increase in endothelial permeability. Further studies are underway to discern isoform-specific mechanisms of beta-arrestins in the termination of VEGFR2 signal transduction.

208/B151

Thrombomodulin as a Plasminogen Receptor to Mediate HUVECs Cell Migration.

P. Chen[1], B. Chang[2,3], C. Kuo[1], G. Shi[2,3], H. Wu[2,3]; [1]Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan, [2]Department of Biochemistry and Molecular Biology, College of Medicine, National Cheng Kung University, Tainan, Taiwan, [3]Cardiovascular Research Center, National Cheng Kung University, National Cheng Kung University, Tainan, Taiwan

Plasminogen (Plg) is a precursor of plasmin (Plm), which plays important roles in angiogenesis and wound healing. However, the Plg receptor responsible for the pericellular distribution of Plg/Plm is still obscure. Thrombomodulin (TM), a transmembrane glycoprotein, is well-characterized as an anticoagulant function, and also plays some roles in angiogenesis and wound healing. This study aims at investigating the interaction of Plg with TM. The result showed that Plg was directly interacted with recombinant TM domains by ELISA and Far Western analysis. Plg bound to immobilized recombinant TM domains 1, 2, and 3 (rTMD123) with a Kd value of 0.30 μM; and C-Myc and His tags-deleted rTMD123 (dCH-rTMD123) bound to Plg with a Kd value of 0.11 μM by surface plasmon resonance assay. Co-immunoprecipitation assay demonstrated that Plg was associated with TM on human umbilical vein endothelial cells (HUVECs). The interaction of Plg with TM was blocked by TM-specific antibody as determined by flow cytometry and cell adhesion assay. Furthermore, the colocalization of rhodamine-Plg with TM at the leading edge of migrating HUVECs was also observed. Finally, Plg activation and Plg-mediated migration on HUVECs were blocked by specific antibody against TM. Taken together, TM is a receptor for Plg in endothelial cells and participates in the localization of proteolytic activity at the leading edge of migrating endothelial cells, which highlights the potential role of Plg/TM in angiogenesis.

209/B152

Transient Receptor Potential Vanilloid Subtype 1 (TRPV1) of Rat Bone Marrow-derived Stromal Cells (BMSCs) Is Activated by Capsaicin In Vitro.

Y. Iwai, F. Aikawa, Y. Matsuda, M. Nakatsuka, S. Kumabe; Osaka Dental University, Hirakata, Japan

Objective: The present study was undertaken with an aim to examine histological changes and turnover of BMSCs from rat femurs cultured in capsaicin-supplemented conditions, as well as those of the microenvironment in vitro. Materials and Methods: Proliferation, differentiation and TRPV1 expression at the mRNA level (RT-PCR) of conditioned BMSCs, and pH changes and calcium assay of the conditioned culture media were examined; the obtained data were statistically analyzed. Results: Elapsed time-dependent cell proliferation of BMSCs peaking at day 10 was observed in alkaline phase and 37 centigrade microenvironment. Although there were no significant differences of proliferation in different conditions on designated experimental days, day 10 was the turning point of proliferation phase where intermittent administration of 25μM CAP resulted in the least amount of cell proliferation than other culture conditions on day 14. The present RT-PCR study revealed expression of TRPV1 mRNA during 3 to 4 days (either day 3 or day 7) after single or intermittent exposure under 25μM CAP). Conclusions: The results suggest that a longer time span for cell culture is required to uncover how adding capsaicin will change cell proliferation and bone marrow stromal cell differentiation. Also, identification of receptors/trophic factors and mechanism to activate and regulate turnover of the TRPV1 channel in the rat BMSCs is what we should confront in future studies.

210/B153

Interaction of Ibogaine Analogs with the Torpedo Nicotinic Acetylcholine Receptor.

J. Malone[1], H. Arias[2], K. Jozwiak[3]; [1]Arizona College of Osteopathic Medicine, Midwestern University, Glendale, AZ, [2]College of Pharmacy, Glendale, Midwestern Universtiy, Glendale, AZ, [3]Medical University of Lublin, Lublin, Poland

The purpose of the research was to characterize the binding sites for ibogaine analogs on the Torpedo nicotinic acetylcholine receptor (AChR) in the resting and desensitized states. We used [3H]18-methoxycoronaridine ([3H]18-MC) Scatchard-plots using Torpedo AChR membranes, [3H] TCP (a well characterized noncompetitive antagonist) competition binding experiments and Schild-type analysis, analog-

induced binding modulation of the agonist [3H]cytisine, and molecular modeling of the Torpedo AChR ion channel and molecular docking of 18-MC. The results indicated that: (1) there is one (0.86 ± 0.13) high-affinity (Kd = 0.23 ± 0.04 μM) binding site for [3H]18-MC in the desensitized AChR; (2) the affinity (in μM) of each 18-MC congener for the [3H] TCP locus in the desensitized state follows the sequence: 18-MC (0.17 ± 0.01) > 2-methoxyethyl-18-MC (1.3 ± 0.1) ~ 18-methylaminocoronaridine (1.3 ± 0.2) > catharinthine (3.2 ± 0.4) ~ albifloranine (3.2 ± 0.3) > ibogaine (5.4 ± 0.3) > 19-OH-ibogamine (40 ± 2). Whereas, the affinity sequence in the resting state is: 18-MC (12 ± 1) > 18-methylaminocoronaridine (20 ± 2) > catharinthine (48 ± 5) > 2-methoxyethyl-18-MC (82 ± 8) > ibogaine (182 ± 17) > albifloranine (252 ± 24) >>> 19-OH-ibogamine (1.3 ± 0.6 mM); (3) Schild-type analysis suggests that 18-MC interacts with the TCP site in a steric manner; (4) [3H]cytisine binding is enhanced by the 18-MC congeners when the AChR is in the resting but activatable state, but not when the receptor is in the desensitized state; and (5) 18-MC interacts with a domain formed between valine (position 13') and leucine (position 9') rings. The binding and modeling results indicate that the 18-MC binding site overlaps the TCP locus located in the middle of the desensitized ion channel, and that ibogaine congeners may inhibit the AChR by inducing the desensitization process.

211/B154

Regulation of α3β4 nAChRs by Src Tyrosine Kinases in Bladder Innervated MPG Neurons.

N. Kim; Basic Nursing Science, Keimyung University College of Nursing, Daegu, South Korea

α3β4 nicotinic acetylcholine receptors (nAChRs) in major pelvic ganglion (MPG) neurons are thought to mediate a major role in pelvic autonomic synaptic transmission. Src family kinases (SFKs), one of the groups of tyrosine kinases, regulate the activities of a variety of neuronal channels including AChRs, which are expressed ubiquitously in many cell types but are especially abundant in neuronal tissues. Nevertheless, the potential roles of nAChRs by STKs are poorly understood. Therefore, we examined the role of phosphorylation by STKs using electrophysiology, calcium imaging, RT-PCR technique and kinase assays in bladder innervated MPG neurons. PP2, a selective Src-kinase inhibitor, attenuated the ACh-induced peak currents and [Ca2+]i in MPG neurons, whereas PP3, an inactive analogue, had no effect. Conversely, ACh-induced inward currents were significantly augmented with an inclusion of sodium orthovanadate (200 μM), a protein tyrosine phosphatase (PTPase) inhibitor. In the tyrosine kinase assay, PTK activity was also significantly inhibited by treatment with 10 μM PP2, but not by PP3. RT-PCR analysis showed that, among families of Src kinases, Src-2 kinase was expressed in high amounts in MPG neurons. Overall these data suggest that the activities of α3β4 AChRs are functionally regulated by the opposing actions of SFKs and PTPases in bladder innervated rat MPG neurons. "This work was supported by the Korea Science and Engineering Foundation (KOSEF) grant funded by the Korea government(MEST) (No. RO1-2008-000-11722-0)"

212/B155

Multiple P2Y Receptors in the 3T3-L1 Preadipocyte.

J. Baik, M. Kim, I. Kong; Department of Physiology, Yonsei Univ. Wonju College of Medicine, Wonju, South Korea

Extracellular ATP elicits diverse physiological effects by binding to the G-protein-coupled P2Y receptors on the plasma membrane. In addition to the short-term effects of extracellular nucleotides on cell functions, there is evidence that such purinergic signaling can have long-term effects on cell proliferation, differentiation and death. The 3T3-L1 cell line derived from mouse embryo is a well-established and commonly utilized in vitro model for adipocytes differentiation and function. However, the distributions and roles of P2Y subtypes are still unknown in the preadipocyte. In this study we identified the distributions and roles of P2Y subtypes in preadipocyte using Ca2+ imaging and real-time-PCR. ATP (EC50=224±0.06 uM, n=9) increased the [Ca2+]i in a concentration-dependent manner. ATP increased Ca2+ in absence and/or presence of extracellular Ca2+. Suramin, non-selective P2Y blocker, largely blocked the ATP-induced Ca2+ response. U73122, a PLC inhibitor, completely inhibited Ca2+ mobilization in 3T3-L1 cells. The mRNA expression by real-time-PCR of P2Y subtypes was P2Y2: P2Y5: P2Y6 = 3.13: 38.97: 1. The relative expression of P2Y subtypes was markedly changed particular in differentiated adipocytes attaining a newly Go/G1 gene. In conclusion, we showed that P2Y5 subtype was a dominant P2Y receptor in preadipocyte, and multiple P2Y receptors may partly contributes to the adipogenesis.

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Immunoprecipitation of a Photoaffinity Crosslinked 125I-TP508S Receptor Complex Suggests That TP508 Binds to an NPAR Receptor Distinct from Integrins αvβ3 and α5β1 or Known PAR Thrombin Receptors.

J. S. Bergmann, D. Carney; Biochem and Mol Bio, Univ Texas Medical Branch, Galveston, TX

TP508 (Chrysalin® or rusalatide acetate) is a non-proteolytic synthetic 23 amino acid peptide representing the domain of thrombin thought responsible for high-affinity thrombin-receptor binding and stimulation of nonproteolytic mitogenic signals. TP508 stimulates revascularization and tissue repair in animal models and is currently being evaluated in human clinical trials for acceleration of fracture repair and treatment of diabetic foot ulcers. Because TP508 initiates cellular effects distinct from those of proteolytically active thrombin, we hypothesized that TP508 acts through a non-proteolytic thrombin receptor (NPAR) that is distinct from the cloned proteolytically activated receptors (PAR1-4). Others have speculated, however, that TP508 may activate integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$, or bind to one of the PAR receptors. To answer these questions, we developed procedures to crosslink ^{125}I-TP508S to its receptor using the heterobifunctional, photoactivatable cross-linker Sulfo-SANPAH and showed the specificity of this crosslinking. To immunoprecipitate (IP) this complex, polyclonal antibodies were produced against the first half (AGYKPDEGKRGDAC [N-terminus]) and second half (KRGDACEGDSGGPFV [C-terminus]) of TP508 and the IgG was purified. Only the antibodies made against the C-terminus half of TP508 were able to IP a ^{125}I-TP508S-receptor complex from human (WI-38) and mouse (B11C) fibroblasts, and from human coronary artery endothelial cells (HCAE). SDS gel electrophoresis followed by autoradiography of these complexes shows a single ^{125}I-TP508S-receptor complex of 108±1 kDa, n=3±SEM (reduced) and 109±5 kDa, n=2±SD (nonreduced). Attempts to IP the ^{125}I-TP508S-complex using antibodies known to IP PAR1-4 or integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ failed. These antibodies, however, were able to IP their target proteins from iodinated cell surfaces. These data suggest that TP508 binds to a receptor other than PAR1-4 or integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$. Efforts are currently underway utilizing these techniques to positively identify what may be a unique TP508 receptor. (Supported by OrthoLogic Corp, Tempe AZ)

214/B157

Estrogen Alters Protein Kinase C Activity in MSC-1 Sertoli Cell Line.

M. P. McGuinness[1,2], E. White[1,2]; [1]Anatomy, Phila College of Osteopathic Medicine, Philadelphia, PA, [2]Center for Chronic Diseases of Aging, Phila College of Osteopathic Medicine, Philadelphia, PA

The objective of these experiments was to determine if Sertoli cells express estrogen receptors on the plasma membrane that alter protein kinase activity. Estrogen receptors (ER) function in the nucleus as ligand mediated transcription factors. In a variety of cells, estrogen receptors associated with the plasma membrane can alter protein kinase activity. Sertoli cells in vivo have been shown to express ERα, but not ERβ. Estrogen can alter gene expression in Sertoli cells, so the nuclear receptors are functional. However, the ability of estrogen to regulate protein kinase (PKC) activity in these cells has not been studied. For these experiments, the MSC-1 Sertoli cell line was used to examine estrogen receptor expression and signal transduction. Both ERα and ERβ were detected in these cells by Western blot analysis. There was a significant increase in the amount of ERα present in cells cultured in the absence of serum compared to cells cultured in the presence of 5% BCS ($p<0.05$). Three-dimensional imaging of immunofluorescence of ERα and ERβ was conducted using z-stack imaging. ERα and ERβ were located in the nucleus and cytoplasm of MSC-1 cells. To determine if some of the receptors were on the plasma membrane, cells were isolated using a magnetic bead separation system. Cells were incubated with primary antibodies to ERα, ERβ, or actin (control). The secondary antibody was conjugated to Biomag particles (Qiagen) and cells were isolated with a magnetic column. Significantly more cells were isolated with antibodies to ERα and ERβ than actin or cells incubated in PBS alone ($p<0.05$). To determine if the surface receptors were active, cells were treated for 2 minutes with 0, 10, 50, 100 or 200 nM 17β-estradiol, or the phytoestrogens, genistein and daidzein. All three treatments increased PKC activity at 200 nM concentration compared to untreated controls ($p<0.05$). In contrast, there was no change in Cyclic AMP dependent protein kinase (PKA) activity. PKA was detected in these cells by Western blot analysis. This is the first indication that estrogens may activate a PKC signaling pathway in Sertoli cells. Continuing to trace this pathway downstream will help define how estrogens affect Sertoli cells.

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Imaging Analysis of the Effect of Netrin-1 on the Distribution of Its Receptor DCC at the Surface of Axon Shafts in Hamster Primary Cortical Neurons.

H. Matsumoto, M. Nagashima; Department of Anatomy, Saitama Medical University, Saitama, Japan

During development, a diffusible axon guidance cue netrin-1 plays a variety of important roles in the correct wiring of the nervous system. For example, it is reported that netrin-1 acts on outgrowth as well as attraction of spinal commissural axons, repulsion of trochlear motor axons, and branch formation of cortical axons. DCC (deleted in colorectal cancer) is known as a membrane receptor for netrin-1, which mediates netrin-1-evoked axon outgrowth and attraction. In rat spinal commissural axons, it is shown that netrin-1 causes translocation of DCC from intracellular vesicular pool to the plasma membrane and that activation of protein kinase A stimulates this netrin-1-induced DCC translocation, enhancing axon outgrowth and extension in response to netrin-1. As for cortical neurons, it remains unknown whether vesicular pool of DCC would exist in the cells, and DCC translocation to the plasma membrane would occur in the axons which are developing branches in response to netrin-1. To address these issues, we examined the effect of netrin-1 on the level and localization of DCC at the cell surface of hamster primary cortical neurons. Quantitative imaging analysis of surface DCC immunofluorescence showed the elevation of DCC level and formation of cluster-like structure of DCC at the surface of cortical axon shafts in response to netrin-1. These changes were abolished when exocytosis was inhibited, suggesting that exocytosis is involved in the regulation of cell surface DCC distribution by netrin-1 in the cortical axon shafts. (Supported by Grant-in-aid for Young Scientists (B) No. 19790157 from the Ministry of Education, Culture, Sports, Science and Technology, Japan.)

216/B159

Dynamic Interactions of Receptors and Heterotrimeric G-Proteins in Dictyostelium Discoideum.

C. Elzie, J. Colby, M. Sammons, C. Janetopoulos; Biological Sciences, Vanderbilt University, Nashville, TN

Extracellular stimuli exercise their effects on eukaryotic cells through serpentine G-protein-coupled receptors (GPCRs) and mediate a plethora of physiological responses. Stimulated receptors activate heterotrimeric G-proteins, consisting of three subunits, α, β and γ. In *Dictyostelium Discoideum*, cAMP binds to the cAMP receptor cAR1, which is coupled to the heterotrimer containing the Gα2 subunit. Until recently, there has been very little in vivo evidence describing how receptors influence the localization of the G-protein complex prior to and after ligand binding. It has been previously shown that the state of the heterotrimer could be monitored by changes in Förster Resonance Energy Transfer (FRET) between the α2 and β-subunits of Dictyostelium discoideum. We now report the kinetics of G-protein activation as a loss of FRET prior to and after cAMP addition using total internal reflection fluorescence microscopy (TIRFM). We also performed photobleaching experiments to measure G-protein recovery times. Lastly, Gα2-subunit localization was analyzed in wild-type and mutant cell lines lacking various signaling components. Our results show that inactive and active G-proteins cycle between the cytosol and inner leaflet of the plasma membrane. These data suggest that cAR1 activation slows the α2-subunit's membrane off-rate, while simultaneously promoting βγ-subunit disassociation.

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Melanin Concentrating-Hormone Signals Actin Depolymerization in 3T3-L1 Pre-adipocytes.

S. Portwood, L. Shum, L. Cook; Biological Sciences, The College at Brockport, SUNY, Brockport, NY

Melanin concentrating hormone (MCH) binds and activates two G protein-coupled receptors involved in the control of appetite and energy expenditure in mammals. MCH receptor 1 interacts with two cytoskeleton-binding proteins in vitro. One of these proteins, periplakin, has been shown to contribute to the desensitization of MCH signaling events by displacing the interacting G protein. While periplakin is an actin- and intermediate filament-binding protein, MCH is not known to signal cytoskeletal rearrangements. This study asks whether MCH receptors mediate actin cytoskeletal rearrangements in response to hormone binding. 3T3-L1 pre-adipocytes, endogenously expressing MCH receptors, were treated with MCH for varying times, fixed, and actin fibers were stained with Alexa Fluor phalloidin. Using fluorescence microscopy,

cells were categorized as 1) having prominent actin stress fibers, 2) being round with many plasma membrane extensions, or 3) being small and round in blinded experiments. Pharmacological agents were used to dissect the contributions of two downstream effectors of Gq, phospholipase C and ADP-ribosylation factor 6, as well as the relative contributions of MCHR1 versus MCHR2 on MCH-mediated actin rearrangements. A small, but statistically significant change in actin morphology was observed after exposure to MCH for a little as 2 minutes showing that MCH does indeed signal to the cytoskeleton in this cell line. Phospholipase C activators mimic this response on a similar time course suggesting it is a major participant in this signal transduction pathway.

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A2B Adenosine Receptor Negatively Regulates NFκB Activation through Physical Interaction with p105.

Y. Sun[1], W. Lam[1], Y. Duan[1], A. Quintana[1], F. Sun[1], Z. Wu[2], P. Huang[1]; [1]Biology, HKUST, Hong Kong, China, [2]Biochemistry, HKUST, Hong Kong, China

A2B adenosine receptor is a typical G protein coupled receptor and modulates a wide array of cellular processes, including immune response, vasodilatation, and cell growth, upon adenosine binding. Some recent studies indicated that A2B adenosine receptor also function as a negative regulator of inflammation without adenosine binding. To better understand the role of A2B adenosine receptor in inflammation, we searched for its potential novel binding partners. Using a yeast two-hybrid approach, we isolated the transcription factor NFκB1/p105 interacted specifically with the C-terminus of A2B adenosine receptor. The A2B-p105 association was further confirmed in mammalian cells by GST pull down and co-immunoprecipitation assays. In addition, we found that the 497-542aa region and PEST domain of p105 was necessary for its interaction with A2B adenosine receptor. Expression of A2B adenosine receptor increased p105 protein expression without affecting p105 mRNA level. Furthermore, A2B adenosine receptor inhibited NFκB activation induced by IκB kinase β. Depletion of A2B adenosine receptor by RNA interference reversed its effect on IκB kinase β-mediated NFκB activation. Taken together, our results indicated that A2B adenosine receptor negatively regulated NFκB activation by physically interacting with p105 and thereby blocking its degradation. The investigation of detailed mechanism of A2B adenosine receptor regulating p105 expression in vitro and in vivo is under way. Supported by Hong Kong Research Grants Council Grants HKUST6468/05M and 661008.

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Differential Functions of Semaphorin 6D on Cerebellar Granular Precursors and Medulloblastoma Cells.

N. Thomasset[1], P. Massoma[1], N. Chounlamountri[1], C. Meissirel[1], V. Rogemond[1], T. Toyofuku[2], A. Kumanogoh[2], J. Honnorat[1]; [1]INSERM U842, Faculty of Medicine, Lyon, France, [2]Dept of Molecular Immunology, Osaka University, Osaka, Japan

During development, nervous progenitors are responsive to microenvironment. Precisely, under guidance cues they migrate on long distances to their specifics targets where they fully differentiate. During tumoral process, cell migration is often impaired leading to invasive growth. Semaphorins, first described as guidance cues for axons have been recently involved in cell migration, apoptosis, nerve regeneration and tumor progression. Our group works on semaphorin 6D (Sema6D), first described in the col- lapse of DRG neurons and migration of embryonic cardiac cells (Qu et al., J. Biol. Chem. 2002; Toyofuku et al., Genes Dev. 2004). Our objective is to explore the role of Sema6D on granule cells precursors (GCPs) compared to Medulloblastoma cell lines DEV and DAOY originated from cerebellum tumors. We investigated the functional response of physiological GCPs and DEV and DAOY tumoral cells under Sema6D. Using a TUNEL and caspase 3 active assays, our results showed a 30% increase of GCPs apoptosis (92 ± 2.7) of apoptosis when seeded with cells expressing Sema6D versus 31 ± 1 with control cells (n=4; p<0,001). Using a chemiotaxis assay, DEV cells migration is significantly increased when they are seeded with Sema6D (22.3 ± 2.3) compared without Sema6D (12 ± 0.7; p<0,01). In contrast the migration of GCPs and DAOY cells are not affected. In addition, we investigate the expression of sema6D, of its receptor and signaling molecules such as Collapsin Response Mediator Proteins (CRMPs) in GCPs, DEV and DAOY cells. In fact, CRMPs are involved in migration and apoptosis of granular precursor cells (Ricard et al., J. Neurosc. 2001, Charrier et al., Genes to cells,2006). Our data showed a differential expression of Sema6D and of plexin A1, its main receptor, in granular cells compared to DEV cells as well as a decrease of CRMP1 expression. These results emphasize a different effect of microenvironmental molecule (Sema6D) on precursor functions. Cell response to Sema6D could be depending on the commitment of the progenitor cells or on the tumoral process.

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Knockout Mutants of Two Annotated GPCR Genes in Tetrahymena Both Show Altered Responses to Divalent Cations.

T. L. Lampert, C. M. Diederich, A. L. Bennett, T. M. Hennessey; Biological Sciences, University at Buffalo, Amherst, NY

Tetrahymena are motile, unicellular eukaryotes that integrate sensory information in the form of depolarizing and hyperpolarizing influences as they swim. In general, chemorepellents cause membrane potential depolarization, slower swimming and ciliary reversals (avoiding reactions) while attractants cause hyperpolarization and faster forward swimming. Examples of chemorepellents are many classical tastants and odorants, several types of polycationic compounds, some oxidants, specific nucleoside triphosphates like ATP and GTP and many types of depolarizing cations. We have identified a number of annotated GPCR sequences in the Tetrahymena Genome Database and have begun to construct knockout mutations of each to determine which of these stimuli are detected by each GPCR. One knockout mutant, called G8, has altered responses to most divalent cations but normal responses to all of the other stimuli tested. While wild type cells avoid divalent ions such as Ni++, Cd++, Zn++ and Cu++ by swimming away from a drop of these ions, the G8 knockout mutant shows attraction and aggregation within the drop. Therefore, this mutation changed several toxic metal chemorepellents into chemoattractants, The G8 mutant is a stable macronuclear knockout in which the coding sequence (TTHERM_01055530) was disrupted with the neo3 construct. A knockout mutant of another annotated GPCR (TTHERM_00925490), called G6, produces a different phenotype. While the G6 mutant has near-normal responses to most stimuli tested, their response to Ba++ is quite different. Under Ba++ conditions that cause wild type to show avoiding reactions and swim very slowly, the G6 mutants are much more motile. We have confirmed the specificities of both the G6 and G8 knockouts with combined Southern blot, quantitative PCR and standard PCR analysis. We propose that these two putative GPCRs may be involved in external divalent ion recognition and avoidance, either as ion channels or as divalent ion receptors. Supported by NSF grant MCB-0445362 to T.M.H.

Membrane Domains and Polarity (221-244)

221/B164

Ankyrin-G and Beta 2-spectrin Coordinate Cargo Recognition and Basolateral Trafficking in Polarized Epithelial Cells.

K. Abdi, J. Q. Davis, K. Kizhatil, V. Bennett; Cell Biology, Duke University, Durham, NC

Protein delivery to the lateral membrane of epithelial cells requires both recognition of basolateral sorting signals and assembly onto motile vesicular or tubular-vesicular carriers. Here we describe a direct role for ankyrin-G in targeting to the basolateral domain through its ability to read diverse sorting signals in basolateral proteins. Ankyrin-G binds to the basolateral VSV-G but does not bind to the apical hemmaglutin (HA) transmembrane proteins. A single C/Y mutation in HA confers both basolateral targeting and a gain of binding to ankyrin-G. Depletion of ankyrin-G or B2-spectrin inhibits the delivery of E-cadherin and NKA to the lateral membrane during membrane biogenesis. Experiments using temperature-sensitive VSV-G result in either a block in transport to the basolateral domain or a delay in transport from ER to Golgi during depletion of B2-spectrin or ankyrin-G respectively. B2-spectrin requires ankyrin-G, since a B2-spectrin mutant lacking ankyrin-binding is incapable of rescuing B2-spectrin-depleted cells. Several experiments suggest that B2-spectrin is a key orchestrator of vesicle trafficking. We have visualized GFP-tagged B2-spectrin on motile tubular-vesicular carriers that co-localize with Rab11 and appear to emerge from the trans-Golgi network. Full-length B2-spectrin binds directly to inositol lipids, PI(4,5) and PI(3,4), through its pleckstrin homology domain (PH). Moreover, a point mutation within the PH domain greatly diminishes its capacity to bind PI lipids in-vitro and completely blocks basolateral targeting of B2-spectrin. The PH domain mutant also fails to form tubular-vesicular structures. The motor complex responsible for trafficking through B2-spectrin is likely to include dynactin/dynein, since depletion of B2-spectrin from epithelial cells eliminates cortical localization of Arp1 and nearly eliminates its filamentous intracellular staining. A mutation in B2-spectrin within the proposed binding site for Arp1 leads to the generation of small non-motile B2-spectrin coated vesicles when expressed into epithelial cells. We suggest that ankyrin-G and B2-spectrin collaborate to provide both cargo recognition and vesicle delivery to the lateral membrane of epithelial cells.

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Phosphorylation of Rab11-FIP2 Regulates Polarity in MDCK Cells.

L. A. Lapierre[1,3], C. M. Caldwell[1,3], K. M. Avant[1,3], J. Schafer[1], N. A. Ducharme[2,4], J. Goldenring[1,2,3]; [1]Surgery and the Epithelial Biology Program, Vanderbilt Univ. Medical Center, Nashville, TN, [2]Cell and Developmental Biology, Vanderbilt Univ. Medical Center, Nashville, TN, [3]Nashville VA Medical Center, Nashville, TN, [4]Institute Molecular Medicine, UTHSCH, Houston, TX

The Rab11 effector, Rab11-Family of Interacting Proteins 2 (Rab11-FIP2) is involved in apical recycling through its interactions with Rab11a and Myosin Vb. We have previously shown a second role for Rab11-FIP2 in the establishment of polarity in MDCK cells through phosphorylation of serine 227 by Par-1b/EMK1/ MARK2. We have now expanded that work into MDCK cells grown both on Transwell filters and in 3D matrigel cultures and investigated the effect of Rab11-FIP2 serine 227 phosphorylation mutants on the lateral junctions and cell polarity. The non-phosphorylatable Rab11-FIP2(S227A) mutant exhibited a defect in establishing polarity in the 3D cultures. While the cells formed into spherical colonies, they did not hollow out the center and form cysts. The putative pseudophosphorylation Rab11-FIP2(S227E) mutant, had a much more dramatic phenotype, exhibiting multiple lumens. A change in the composition of the lateral junctions was also observed in the Rab11-FIP2(S227E)-expressing cysts. While p120 and K-cadherin remained at the adherens junction, the majority of the E-cadherin was lost. At the tight junction we observed the loss of occludin, but the retention of ZO-1. Reductions in E-cadherin and occludin were confirmed by Western blotting. Similar results were observed in Rab11-FIP2(S227E) cells grown on Transwell filters. No drop in TER was detected in the monolayers grown on transwells and GP135 remained in the apical membrane of the cells within the cysts, indicating no overt loss of junctional function. These results indicate that Rab11-FIP2 plays an important role in regulating the composition of both adherens and tight junctions though phosphorylation of serine 227 and thereby is intimately involved in the regulation of polarity in epithelial cells.

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Rab14 Modulates Arf1 Localization and COPI Recruitment at the TGN.

K. Kitt[1,2], D. A. Elliott[1], J. Wilson[1]; [1]Cell Biology and Anatomy, University of Arizona, Tucson, AZ, [2]Biology, Stanford University, Stanford, CA

Formation of vesicle carriers from the trans-Golgi network (TGN) for targeting of newly synthesized proteins requires Arf1 and coat proteins. Arf1 and clathrin mediate vesicle budding at the TGN and Arf1 and COPI are known to perform similar functions at the cis-Golgi. However, although COPI has been localized to the TGN and endosome, the functional role of Arf1 and COPI at the TGN remains unknown. Coat proteins together with their effectors can define functional membrane domains; here, we show that the small GTPase Rab14 mediates Arf1 localization and COPI recruitment at the TGN. We demonstrate that Rab14 localizes to distinct domains of the TGN compared to the basolateral/TGN resident protein, TGN38. Overexpression of the GDP-mutant form of Rab14 increases the amount of COPI and GBF1 at the TGN without affecting clathrin heavy chain localization. Although Rab14 does not bind Arf1 directly, expression of Rab14-S25N enriches the binding of the Arf1-GDP-bound mutant to the TGN. These data suggest that Rab14 defines a membrane domain of the TGN that regulates Arf1 activation and COPI localization at the TGN.

224/B167

Afi1p Functions as an Arf3p Polarization Docking Factor for Development of Polarity.

K. Chen, P. Tsai, F. Lee; Molecular Medicine, NTU, Taipei, Taiwan

ADP-ribosylation factors (Arfs) are highly conserved small GTPases and are critical components of vesicle trafficking and actin reorganization. Yeast Arf3p, in spite of its similarity to mammalian Arf6, is not required for endocytosis, but is involved in polarity development. In this study, we identified an Arf3p-interacting protein 1 (Afi1p), which, through its N-terminal conserved region, interacts

with Arf3p in a GTP-dependent manner. Afi1p is cell-cycle regulated and distributed asymmetrically at the plasma membrane. Afi1p is required for polarized distribution of Arf3p, but not of an Arf3p guanine nucleotide-exchange factor, Yel1p. However, Afi1p is not required for targeting of Arf3p or Yel1p to the plasma membrane. Like arf3 mutant yeast, afi1 mutant yeast exhibited an abnormal budding pattern and partially delayed actin patch polarization. An Afi1p, 38KLGP4A-Afi1p, mutated at the Arf3p-binding region, loses its ability to interact with Arf3p and maintain the polarized distribution of Arf3p. Although 38KLGP4A-Afi1p still possessed a proper polarized distribution, it lost its ability to rescue actin patch polarization in afi1 mutant cells. Our findings demonstrate that Afi1p functions as an Arf3p polarization-specific adapter and participates in development of polarity.

225/B168

Annexin A2 and Lipid Microdomains Regulate Matrix Vesicle-associated ALP Activity Required for Osteoblastic Mineralization.

S. Nielsen-Preiss[1], J. Gillette[2]; [1]Cell Biology and Neuroscience, Montana State University, Bozeman, MT, [2]NICHD, CBMB, National Institutes of Health, Bethesda, MD

Osteoblastic mineralization is a highly regulated process that performs a crucial role in bone formation during development and remodeling. Our laboratory has previously reported an increased rate of osteoblastic mineralization upon the overexpression of annexin A2. Our data indicated that annexin A2-mediated mineralization was, in part, due to an increase in alkaline phosphatase activity associated with lipid microdomains. However, how annexin A2 and lipid microdomain-associated alkaline phosphatase activity regulated the extracellular process of mineralization remained unclear. The following study proposes a mechanism by which annexin A2 affects the mineralization process in osteoblastic cells. Data described herein suggest that mineralization of osteoblastic cells occurs through the production of classically defined matrix vesicles, similar to those observed in chondrocytes. We demonstrate that isolation of mineralization-competent matrix vesicles is enhanced upon annexin A2 overexpression and reduced in annexin A2 knock-down studies. We obtain comparable results when evaluating the alkaline phosphatase activity associated with lipid raft microdomains under both expression conditions. Therefore, we show additional data to support the hypothesis that membrane microdomains constitute a precursor for matrix vesicles. Treatment with methyl-β-cyclodextrin, a cholesterol-sequestering agent, reduces the alkaline phosphatase activity in both matrix vesicles and lipid microdomains, suggesting similarities in membrane structure. Alterations of cell surface morphology, consistent with matrix vesicle formation, are also observed using scanning electron microscopy. These studies suggest that annexin A2 enhances the mineralization competence of osteoblastic matrix vesicles at least in part, by increasing vesicle- and lipid microdomain-associated alkaline phosphatase activity. From these data we suggest that lipid microdomains may serve as recruitment and organization sites for proteins destined for matrix vesicles.

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Role of FGD6 in Actin Ring Formation and Organelle Dynamics in Resorbing Osteoclasts.

A. Espirito Santo, T. Heckel, B. Hoflack; Biotechnology Center, Dresden, Germany

Osteoclasts (OCs), multinucleated cells derived from the monocyte/macrophage lineage, are critical for bone remodelling. Their resorption function depends on the organization of their actin cytoskeleton into a specialized structure - the sealing zone. OCs attach to the bone matrix at the particular site where the bone will be resorbed, they are activated and initiate the resorption cycle, that includes the relocalization at the sealing zone of secretory and endocytic organelles required for the secretion of hydrolytic enzymes or the uptake of digested material, respectively. The sealing zone is formed by condensing podosomes into a highly dymanic podosomal belt upon contact with bone surface. The podosomal belt transmits polarity cues for the targeted delivery of lysosomal proteins to the ruffled border. Podosome turnover, actin ring formation and endocytosis are regulated by RhoGTPases, whose activity is catalyzed, amoung others, by RhoGEFs (guanine nucleotide-exchange factors) in response to extracellular stimuli. We have shown that, during osteoclastogenesis, 8 RhoGEFs, including FGD6, are drastically upregulated. Here, using recombinant adenovirus, interference RNA, chemical inhibition and fluorescence microscopy, we show that FGD6 is a Src target that regulates sealing zone formation and may play a role in the recruitment of Rab5-positive early endosomes (EEs) to the sealing zone. We next analyze the different domains of FGD6 in order to understand how they contribute to actin cytoskeleton organization and to the recruitment of Rab5 positive EEs. Our data indicates that, in HeLa cells and OCs, the PH and FYVE domains, but not the RhoGEF domain, are sufficient for FGD6 association with EEs and actin filaments. In OCs, the PH domain is sufficient for localization to podosomes. In conclusion, FGD6 regulates sealing zone formation and recruitment of EEs to this area and thus may play a crucial role in OC resorption.

227/B170

The Polybasic Region Is Not Essential for Membrane Binding of the Influenza Virus Matrix Protein M1 in Cells.

B. Thaa[1], A. Herrmann[2], M. Veit[1]; [1]Faculty of Veterinary Medicine, Institute for Immunology and Molecular Biology, Free University Berlin, Berlin, Germany, [2]Molecular Biophysics, Humboldt University, Berlin, Germany

The matrix protein M1 forms a proteinaceous layer underneath the lipid bilayer of influenza virus particles and interacts with the glycoproteins hemagglutinin (HA) and neuraminidase (NA) and with the viral genome. In the infected cell newly synthesized M1 transports the viral genome from the nucleus to the plasma membrane, where the interactions with HA and NA supposedly lead to a concentration of the viral components, which is a prerequisite for virus budding. Hence, M1 is thought to be the key organizer of virus assembly. Furthermore, M1 has an intrinsic lipid-binding activity, the focus of the current study, which targets the protein to cellular membranes. Based on liposome experiments it has been proposed that membrane binding is mediated by a stretch of basic amino acids seen at the surface of the atomic structure of the N-terminal part of M1. Surprisingly, this assumption was never tested experimentally inside cells. To analyze its intrinsic membrane-binding activity *in vivo*, M1 has to be expressed in the absence of other viral components, but in that case the protein accumulates in the nucleus. Furthermore, removal of the nuclear localization signal of M1 is not a reasonable approach since it overlaps with the proposed membrane binding region. We have solved this problem by fusing a nuclear export signal (NES) obtained from the Rev protein of HIV to the C-terminus of YFP-M1 and show that the resulting construct is excluded from the nucleus and mainly membrane-bound in transfected cells. Subcellular localization features of YFP-M1-NES were assessed by immunofluorescence and were found to be identical to the wildtype protein, providing evidence that all targeting features except nuclear localization had remained unchanged. The extent of membrane

association was tested biochemically by membrane binding and floatation assays. Exchanging the polybasic region in YFP-M1-NES by alanines did not prevent the protein from binding to membranes, nor did the further replacement of adjacent hydrophobic and possibly membrane-inserting residues. Thus, M1 probably binds to membranes via multiple binding sites, allowing the protein to variably attach to several interaction partners and hence organize virus assembly.

228/B171

PIPs Modulate the Sterol Affinity of Oxysterol Binding Proteins at Membrane Contact Sites.

T. A. Schulz, R. E. Chung, W. A. Prinz; NIDDK, National Institutes of Health, Bethesda, MD

Membrane contact sites (MCS) are zones of close apposition between two organellar membranes, and are thought to be sites of interorganellar lipid transfer. They may be enriched in certain specific proteins and lipids, but so far, few components of MCSs have been definitively identified. Using the yeast Saccharomyces cerevisiae as a model, we demonstrate that several proteins related to lipid transport, metabolism and binding are localized to junctions between the plasma membrane (PM) and endoplasmic reticulum (ER). These include several oxysterol binding protein homologs (Osh2p, Osh3p, Osh6p and Osh7p), a family of proteins defined by a core sterol-binding domain. We found that the core domain of all seven yeast Osh proteins is capable of interacting with multiple membranes, monomerically inducing aggregation of liposomes. Furthermore, using purified yeast organelles in vitro we found that Osh6p and Osh7p cause microsomes to associate with both lipid droplets and inside-out plasma membrane vesicles. Even more intriguingly, we found that the ability of Osh4p to extract sterol from artificial liposomes is influenced by the addition of a second, separate population of liposomes containing species of phosphatidylinositol phosphates (PIPs). PI(4,5)P2 in the secondary liposomes decreases the half-time of sterol extraction by an order of magnitude, and slightly enhances the maximal amount of extracted sterol. On the other hand, PI(4)P reduces the affinity of Osh4p for sterol in a concentration-dependent manner. This data represents the first known instance of a single lipid-binding domain interacting with multiple membranes simultaneously, where the species of lipid at one binding site can modulate the affinity for the ligand at a second site. We propose a model in which Osh4p extracts sterol from MCSs where sterol is enriched in one membrane and PI(4,5)P2 is enriched in the other, and can deliver it to sites where one of the apposed membranes is enriched in PI(4)P. We also identify other lipid-binding proteins at PM-ER contact sites, and discuss their significance.

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Identification of a Novel Regulator of Phosphatidylinositol 4-phosphate Synthesis at the Plasma Membrane.

D. Baird, C. Stefan, S. Emr; Weill Institute for Cell and Molecular Biology & Molecular Biology and Genetics, Cornell University, Ithaca, NY

Phosphoinositide (PI) lipids are essential for cell division, polarity, signal transduction, and membrane trafficking. Among the four distinct PIs identified in yeast, phosphatidylinositol 4-phosphate (PI4P) is critical for polarization of the actin cytoskeleton, activation of the Pkc1 pathway, and secretion. At the plasma membrane (PM), PI4P's general impact is often credited to its role as the PI(4,5)P_2 precursor; however, several functions unique to PI4P have emerged. For example, recent studies have implicated PI4P as a regulator of lipid metabolism; potentially through its ability to directly interface with oxysterol binding proteins (OSBP) and the yeast VAP-A homolog Scs2. Signaling to these effectors requires dynamic turnover and localized synthesis of PI4P by the appropriate lipid kinases and lipid phosphatases. We are interested in identifying the various components regulating PI4P turnover at the plasma membrane and its subsequent affect on cellular function. The majority of plasma membrane PI4P in *Saccharomyces cerevisiae* is generated by the essential PI 4-kinase Stt4. Interestingly, Stt4 clusters into cortical assembly sites we term PIK (PI Kinase) patches containing multiple copies of the PI 4-kinase. To understand the architecture, regulation, and function of PIK patches, we characterized a novel, essential Stt4-interacting protein, Ypp1 (YGR198w). We show that Ypp1 co-localizes with Stt4 at PIK patches where it regulates the location, stability, and activity of this lipid kinase. We demonstrate that Ypp1 interacts with Stt4 and is necessary for recruitment of the PI 4-kinase to the PM and for the correct assembly of multiple copies of the kinase and other regulatory proteins at the PIK patch. We propose that PIK patches provide platforms for the co-ordinate regulation of PI kinases and the localized control of PIP synthesis.

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GPI-anchored Protein's Complex and Membrane Skeleton in Human Neural Progenitors as Revealed by Electron Microscopy.

N. Morone, F. Wakui, T. Kohno, Y. Kimori, S. Yuasa; Department of Ultrastructural Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Japan

The pathogenesis of neurodegenerative diseases has been mostly understood by the accumulation of proteinic self-aggregates, or the formation of short amyloid filaments. We have examined the membrane structure and the formation of the molecular complex of the cellular prion protein (PrPC), a glycosylphosphatidylinositol (GPI)-anchored protein, in the human neuronal progenitors by using electron microscopy. Though it is well known that the PrPC localizes on the outer surface of the plasma membrane, caveolae or clathrin-coated pits in fibroblastoids, the relationship with the part of the cellular skeleton in the range of tens of nanometers from the plasma membrane (which called the "membrane skeleton") is unknown. Therefore, the function of PrPC and the structural change from PrPC to the pathogenic prion (PrPSc) might remain to be unclear. In this study, we showed that the PrPC could localize on the mitochondria or the actin cytoskeleton in the human neural progenitor, with a molecular chaperonin and the 14-3-3ζ, which detected in the cerebrospinal fluid of Creutzfeldt-Jacob disease (CJD). The formation of these protein-complexes could be partially affected by actin depolymerization and cholesterol content. We also have firstly visualized the neural membrane skeleton of the human neural progenitors by the rapid-freeze, deep-etch, and platinum-replica electron microscopy, which covers the entire cytoplasmic surface and is closely linked to clathrin-coated pits. Since these filaments have a specific stripped pattern with a 5.5 nm periodicity, the neural membrane skeleton primarily consists of actin filaments. These finding may be useful to understand a cause of CJD, the conformation change of the PrPC into the PrPSc in the neural system.

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Characterization of Bile Salt Resistant Microdomains in Rat Liver Canalicular Plasma Membranes.

C. Guyot, G. A. Kullak-Ublick, B. Stieger; Department of Medicine, University Hospital, Zurich, Switzerland

Canalicular lipid (phosphatidylcholine (PC) and cholesterol) secretion requires the coordinate action of several ATP binding cassette (ABC) transporters: The bile salt export pump (Bsep) secretes bile salts (BS), which release PC from the plasma by their detergent action in conjunction with the PC translocator multidrug resistance protein 2 (Mdr2). In the canaliculus, PC and BS form mixed micelles, which act as acceptors for cholesterol presented by Abcg5/g8. The cLPM is enriched in sphingomyelin and biophysical experiments demonstrated a preferential interaction of sphingomyelin with cholesterol. Importantly, incubation of isolated cLPM vesicles with taurocholate in vitro leads to a preferential release of PC into the supernatant. Hence, in analogy to detergent insoluble lipid rafts, the cLPM may contain BS insoluble rafts. Objectives: To test whether incubation of cLPM with different BS results in the generation of bile salt resistant microdomains (BRMs). Methods: Highly purified rat cLPM were extracted at 4°C with 3 BS with increasing CMC (taurodesoxycholate (TDC) < taurocholate (TC) < glycocholate (GC)) and floated in sucrose step gradients for. Gradient fractions were then used for Western blotting and lipid analysis. Results: Increasing TC concentrations from below to above CMC demonstrated that a TC concentration around or higher than CMC was required for complete cLPM solubilisation. Extraction of cLPM with micellar BS concentrations led to the formation of BRMs containing the rafts markers reggie-1, -2 and caveolin-1 and canalicular ABC transporters. 31% of Bsep was found in TC induced BRMs and about 15% in GC and TDC induced BRMs, 11% of total Abcg5 was found in GC induced BRMs and 20% in TC and TDC induced BRMs, and about 50% of total Mdr1 was found in the three different BRMs. Lipid analysis shows that BRMs contain mainly cholesterol, PC, phosphatidylethanolamine and in lower amounts phosphatidylserine and sphingomyelin. Conclusions: These data suggest that cLPM contain BRMs containing typical rafts markers and canalicular ABC transporters. Therefore, it can be speculated that the cLPM contains specific microdomains enriched in biliary lipids, from where lipid secretion into the canaliculus starts.

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Lateral Compartmentation of the Plasma Membrane: Structure and Possible Function of Stable Patchy Domains in Yeast.

G. Grossmann[2], J. Malinsky[1], V. Stradalova[1], W. Stahlschmidt[2], M. Loibl[2], M. Opekarova[3], M. Blazikova[1], W. Tanner[2]; [1]Microscopy Unit, Institute of Experimental Medicine CAS, Prague 4, Czech Republic, [2]Institute of Cell Biology and Plant Physiology, University of Regensburg, Regensburg, Germany, [3]Institute of Microbiology CAS, Prague 4, Czech Republic

It becomes generally accepted that the plasma membrane is laterally compartmented into domains of different lipid and protein composition. So far, we identified nine proteins accumulated in ergosterol-rich 300 nm stable patchy areas of MCC (membrane compartment of arginine permease Can1) in the plasma membrane of S. cerevisiae. These were three transporters with twelve transmembrane domains and six proteins of unknown function with predicted tetraspan structure. In addition, the distribution of cortical patches formed by at least twelve cytosolic proteins was matched with the MCC pattern. Using various electron microscopy approaches, we were able to show that MCC patches are fluorescence microscopy correlates of furrow-like plasma membrane invaginations observed on freeze-fractured cells. The presence of stably curved membranes in these structures is in agreement with the proposed lipid composition of MCC. Among the integral MCC components, Nce102 seems to play a major role in the membrane domain composition. Deletion of Nce102 resulted in the homogenous patterns of MCC-specific transporters; accordant changes in the membrane ultrastructure were detected in this deletion strain. We document that the lack of Nce102 as well as that of Pil1, a primary component of eisosomes, cytosolic structures co-localizing with MCC, accelerate substrate-induced endocytosis and/or turnover of Can1. Proteins essential for classical endocytosis as for example Ede1, Sla2 and Rvs161 do not co-localize with MCC patches. This suggests that proteins within the MCC are protected from degradation, and that in general, lateral segregation of plasma membrane constituents controls protein turnover.

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SBD, a Sphingolipid-binding Fluorescent Probe Traces Microdomain-derived Trafficking Pathways in Living Cells.

E. Lee[1], M. Manna[3], G. Tresset[1,5], D. Zhang[1,4], S. Hebbar[1], S. Steinert[1,2], T. Wohland[3], R. S. Kraut[1]; [1]A-STAR, Singapore, IBN, Singapore, [2]Physikalisches Institut, TU Stuttgart, Stuttgart, Germany, [3]Dept of Chemistry, National University of Singapore, Singapore, [4]Duke/NUS Graduate Medical School, Singapore, Singapore, [5]Laboratoire de Physique des Solides, CNRS Universite Paris-Sud, Orsay, France

The uptake and intracellular trafficking of sphingolipids, which coalesce into plasma membrane microdomains, is associated with many pathological conditions, including virus and toxin infection, lipid storage disease, and neurodegeneration. However, the means available to track the surface behavior and trafficking of sphingolipids in live cells are extremely limited. In order to address this problem, we have developed an exogenous sphingolipid-binding probe, consisting of the 25-amino acid Sphingolipid Binding Domain (SBD) found in the Alzheimer's Aβ peptide (Fantini et al., '02), conjugated by a neutral linker with an organic fluorophore (Hebbar et al, '08; Steinert et al, '08). On both living cells and artificial membranes, SBD prefers raft-like lipid mixtures containing cholesterol and sphingomyelin, and binding is boosted by the presence of gangliosides. Removing any of these components in cells or in membranes limits its binding ability. However, SBD's binding properties are unlike that of a second ganglioside-interacting probe, CtxB. SBD also behaves differently from CtxB in that it does not induce microdomain clustering, when visualized by atomic force microscopy. However, its diffusion at the plasma membrane as measured by fluorescence correlation spectroscopy is distributed bimodally into a slow and a fast population, as has been observed for other raft probes (Hebbar et al, '08; Bacia et al, '04; F. Pinaud and X. Michalet, pers. comm.). Diffusion of the slow population is sped up by extraction of cholesterol and depletion of sphingolipids, consistent with localization in the liquid ordered (raft) phase. SBD is endocytosed independently of CtxB via one of two uptake routes from the plasma membrane, one cdc42- and the other flotillin2-associated. After uptake, SBD is directed to a recycling or a degradative pathway, dependent on the choice of uptake mechanism and the cholesterol content of the cell. Interestingly, SBD trafficking to lysosomes is strongly diverted by intracellular cholesterol overload or depletion, suggesting that SBD may be useful as a diagnostic tool for tracing altered raft trafficking pathways in disease states such as Alzheimer's and the lipid storage diseases.

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Steady State Dynamics of GPI-anchored Protein Nanoclusters in Living Cell Plasma Membrane Is Modulated by Cortical Actin Activity.

D. Goswami[1], K. Gowrishankar[2], M. Rao[2,1], S. Mayor[1]; [1]Cell Biology, National Centre for Biological Sciences-TIFR, Bangalore, India, [2]Raman Research Institute, Bangalore, India

Many putative raft-associated cell surface lipid-tethered proteins exhibit an unusual concentration-independent, cholesterol-sensitive organization of nanoscale-clusters and monomers. To understand the mechanism of formation of these clusters we investigate the spatial distribution and steady-state dynamics of fluorescently-tagged glycosyl-phosphatidylinositol-anchored protein nanoclusters, at the cell surface using high-spatial and temporal resolution Förster's Resonance Energy Transfer (FRET) microscopy. These studies reveal a non-random spatial distribution of nanoclusters, concentrated in optically resolvable domains. Monitoring the dynamics of recovery of fluorescence intensity and anisotropy, we find that nanoclusters are immobile, and the dynamics of interconversion between nanoclusters and monomers, over a range of temperatures, is spatially heterogeneous and non-Arrhenius, with a sharp crossover coinciding with a reduction in the activity of cortical actin. Cholesterol-depletion perturbs cortical actin, and the spatial scale and interconversion dynamics of nanoclusters. Direct perturbations of cortical actin activity also affect the construction, dynamics and spatial organization of nanoclusters. These results provide evidence for a unique mechanism for molecular complexation of cell surface proteins and lipids, whose dynamics and statistical distribution is regulated by the activity of cortical actin.

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FLIM-FRET Studies on Raft-association of Influenza Virus Hemagglutinin: The Pre- and Postfusion Conformations of HA Differ in Their Affinity for Membrane-Rafts.

S. V. Engel[1], S. Scolari[2], N. Krebs[2], T. Korte[2], A. Herrmann[2], M. Veit[1]; [1]Immunology and Molecular Biology, Free University, Berlin, Germany, [2]Biology / Molecular Biophysics, Humboldt University, Berlin, Germany

It has been supposed that the hemagglutinin (HA) of influenza virus must be recruited to membrane-rafts, dynamic liquid-ordered assemblies enriched in cholesterol and sphingolipids, to perform its function in virus budding and membrane fusion. Using fluorescence-lifetime imaging microscopy (FLIM-FRET) of living cells we show strong association of HA, fused at its cytoplasmic tail to the fluorophore Cerulean, with Myr-Pal-YFP, an established marker for inner-leaflet rafts. Clustering is significantly reduced when rafts are disintegrated by cholesterol depletion and when the known raft-targeting signals of HA, the palmitoylation sites and amino acids in its transmembrane region, were removed. Clustering is also diminished when cells are treated with the microfilament disrupting agent cytochalasin D. Thus, both membrane-rafts as well as the actin meshwork lining the inner leaflet of the membrane contribute to clustering. Acceptor-photobleaching FRET reveals that sites of interaction between HA-Cerulean and Myr-Pal-YFP are unequally distributed at the plasma membrane. We also detect FRET at perinuclear regions, probably the trans-Golgi, the intracellular site where membrane-rafts are believed to be generated. Surprisingly, conversion of HA from the pre- to the postfusion conformation by proteolytical cleavage of the molecule into its HA1 and HA2 subunits and subsequent treatment with mildly acidic pH drastically reduces its affinity for membrane-rafts. We speculate that the decrease in the affinity of HA for rafts occurring during or after its membrane fusion-mediating conformational change might be involved in formation or enlargement of the fusion pore.

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Phase-Field Simulations of the Morphological Evolution of Lipid Bilayer Membranes.

C. M. Funkhouser[1,2], F. J. Solis[3], K. Thornton[2]; [1]Biomedical Engineering, University of Michigan, Ann Arbor, MI, [2]Materials Science & Engineering, University of Michigan, Ann Arbor, MI, [3]Integrated Natural Sciences, Arizona State University West, Glendale, AZ

We present a continuum-level simulation method for modeling phase separation (lipid raft formation) and morphological evolution of multicomponent lipid bilayer membranes. Our objective is to investigate how various physical parameters input into the model, such as spontaneous curvature, phase fraction, and interleaflet coupling strength, affect the dynamics and equilibrium morphological phases formed in two-phase lipid bilayer membrane systems. The model applies to membranes with planar background geometries, simulating a nearly planar portion of membrane. The model treats the individual composition of each bilayer leaflet, which determines the spontaneous curvature. The compositions and shape of the membrane are coupled through a modified Helfrich free energy, which includes a term coupling the compositions of the leaflets. The compositional evolution is modeled using a phase-field method and is described by a Cahn-Hilliard-type equation, while the shape changes are described by relaxation dynamics. For nearly planar bilayer systems with each leaflet having the same phase fraction, we find that domains align to reduce the interaction energy, as expected. However, when the coupling effect is stronger, this alignment occurs more quickly and more precisely, showing that the coupling affects the dynamics. For bilayer systems with leaflets having different phase fractions, domains still show a tendency to align, though the phase boundaries on the two leaflets are forced to be offset as a result. This coupling between leaflets is found to heavily influence morphological evolution; in some cases the equilibrium morphological phase observed is very different from what was observed with our simpler monolayer model using similar conditions. We construct a phase diagram of equilibrium morphological phases in the composition space for a few values of the strength of the leaflet coupling. This model has been able to reproduce results found in lipid bilayer experiments regarding interleaflet interactions, including the effect of domain registration across leaflets.

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Cell Surface Protein Distributions Provide Insight into the Physical Basis of Plasma Membrane Lateral Heterogeneity.

S. Veatch, E. Chiang, D. Holowka, B. Baird; Chemistry and Chemical Biology, Cornell University, Ithaca, NY

It is widely recognized that proteins and lipids are heterogeneously distributed on the cell surface, yet little is known regarding the underlying physical principles that give rise to plasma membrane lateral organization. Recently, we characterized robust and dynamic critical fluctuations in isolated plasma membrane vesicles and proposed that critical fluctuations provide a plausible physical basis for <50 nanometer-sized lateral heterogeneity in the plasma membranes of intact cells (1). In order to test this hypothesis, we visualize the submicron

lateral distributions of gold particle-labeled proteins and lipids on the surface of intact, chemically fixed, RBL-2H3 mast cells using scanning electron microscopy (SEM), and we quantify their distributions using a correlation function analysis. Correlation functions are routinely used by physical scientists to decipher interactions in complex systems, and we use them here to validate different possible physical bases of plasma membrane lateral heterogeneity. We find that proteins and lipids in resting cells are significantly correlated at short distances (<30nm) and uncorrelated at long distances (>50nm), consistent with previous studies. In addition, the detailed shapes of experimentally derived correlation functions provide additional information into the organizing principles that give rise to membrane heterogeneity. We evaluate the validity of different proposed mechanisms of membrane organization by fitting our experimental findings to predictions of various models. Preliminary analyses support a critical fluctuation hypothesis, although models involving micro-emulsions and coupling to cytoskeletal components are also investigated. Implications for cellular processes such as signaling will be discussed. 1. Veatch, S. L., P. Cicuta, P. Sengupta, A. Honerkamp-Smith, D. Holowka, and B. Baird. 2008. Critical fluctuations in plasma membrane vesicles. ACS Chemical Biology 3:287-293.

240/B183

Nanoscopic Rearrangement of Outer and Inner Leaflet Membrane Proteins Due to IgE Receptor Cross-Linking.

E. N. Chiang[1], S. Veatch[1], C. Ober[2], D. Holowka[1], B. Baird[1]; [1]Chemistry & Chemical Biology, Cornell University, Ithaca, NY, [2]Materials Science & Engineering, Cornell University, Ithaca, NY

Antigen-mediated cross-linking of immunoglobulin E (IgE) bound to its high affinity receptor FcεRI on mast cells initiates a transmembrane signaling cascade that results in cell activation and exocytotic release of chemical mediators involved in allergic response. Plasma membrane lipids and proteins redistribute as part of this transmembrane signaling process. To understand the functional role of these redistributions, resolution of their size, composition and structure on the nanometer scale is required. We utilize high resolution scanning electron microscopy (SEM) to directly visualize sub-micron membrane domains in intact cell membranes. In our experiments, the distribution of gold-labeled proteins and lipids is analyzed at the surface of intact fixed cells using backscattered electron detection. In parallel, we also observe membrane topography using secondary electron detection. We use a pair-correlation function analysis to quantify protein distributions and parameterized domain size. We have mapped the distribution of a variety of proteins, both related and non-related to the IgE signaling pathway. Using this experimental and quantitative method, we observe dramatic changes in the nano-scale membrane distribution of IgE due to stimulation with multivalent ligands. In resting cells, IgE receptors are clustered into small domains of less than 30nm. Following receptor cross-linking, receptors are redistributed into large domains which are correlated at long length-scales. Additionally, we observe cross-linking dependent rearrangement of several inner leaflet-associated proteins that are implicated in early signaling events. In contrast, outer leaflet GPI-linked proteins are not affected. These findings demonstrate selective nanoscopic reorganization during the initiation of receptor signal transduction.

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Comprehensive Identification of PIP3-regulated Proteomes from C.elegans to H.sapiens by Model Prediction and Live Imaging.

W. Park[1,2], W. Heo[1,2,3], J. H. Whalen[1,2], N. O'Rourke[1,2], H. M. Bryan[1,2], T. Meyer[1,2], M. Teruel[1]; [1]Chemical and Systems Biology, Stanford University, Stanford, CA, [2]Alliance for Cellular Signaling, Stanford University, Stanford, CA, [3]Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, South Korea

Phosphoinositide 3-kinase (PI3K) and its product phosphatidylinositol(3,4,5)-trisphosphate [PIP3] control cell growth, migration and other processes by recruiting proteins with pleckstrin homology (PH)-domains and possibly other domains to the plasma membrane (PM). However, previous experimental and structural work with PH-domains left conflicting evidence which ones are PIP3-regulated. Here we used live-cell confocal imaging of 130 YFP-conjugated mouse PH-domains and found that 20% translocated to the PM in response to receptor-generated PIP3 production. We developed a recursive-learning algorithm to predict PIP3 regulation of 1200 PH-domains from different eukaryotes and validated that it accurately predicts PIP3-regulation. Strikingly, this algorithm showed that PIP3 regulation is specified by amino acids across the PH-domain, not just the PIP3 binding pocket, and must have evolved several times independently from PIP3-insensitive ancestral PH-domains. Finally, our algorithm and live-cell experiments identify the PIP3-regulated proteomes as consisting of 2 C.elegans, 4 Drosophila, and 40 vertebrate proteins.

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Microarray and Proteomics Analysis of Human Tumor Cell Pseudopodia.

J. Shankar[1], A. Messenberg[1], B. Joshi[1], J. Chan[1], L. J. Foster[2], T. M. Underhill[1], I. R. Nabi[1]; [1]Cellualr and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada, [2]Department of Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada

Polarized localization of proteins and RNA to pseudopodia is a critical element of tumor cell motility and thus metastasis. Identification of tumor cell pseudopodia-enriched molecules may identify metastasis-associated genes. Six tumor cell lines [DU145 (Prostrate), MDA-231 (Breast), MDA-435 (Melanoma), HT1080 (Fibrosarcoma), U87 and U251 (Glioma)], out of 15 cell lines screened, projected abundant pseudopodia through 1μm pore filters by confocal imaging and actin labeling. Purity of pseudopodia fractions from these cell lines was determined by the absence of mitochondrial HSP70. A combined bioinformatics and experimental approach was used to identify molecules with the potential to be common biomarkers across various tumor types. For this microarray and mass spectrophotometric analysis performed on purified cell body and pseudopod fractions identified more than 400 upregulated RNA and 200 protein molecules in the pseudopodia with approximately 350 RNA and 70 protein molecules common across the six cell lines. Ingenuity network analysis of common RNA and protein molecules showed that most of the pseudopodial localized molecules have a predominant functional role in cancer and many are involved in glycolysis or signaling. Of these, 8 molecules upregulated in pseudopodia and 2 primarily localized in the cell body fraction were chosen for further study. Real time PCR, western blot and immunofluorescence revealed that 4 out of these 8 molecules were found to represent novel markers of tumor cell protrusions in the various tumor cell lines. A combined gene array and proteomics approach has thus led to the

identification of tumor biomarkers that might be involved in pseudopodia formation, tumor cell motility and metastasis of various tumor types. Supported by Strategic Research Grant on Genomics and Proteomics of Metastatic Cancer from the Cancer Research Society.

243/B186

High-Density Caveolar Formation Just Beneath the Plasma Membrane of the Fully Differentiated Adipocyte as Revealed the Freeze-etch Electron Microscopy.

F. Wakui, T. Kohno, Y. Kimori, K. Hattori, S. Yuasa, N. Morone; Department of Ultrastructural Research, National Institute of Neurosciences, National Center of Neurology and Psychiatry, Kodaira, Japan

Caveola, a kind of membrane invagination, was found on the cytoplasmic surface of the cell membrane of the blood capillary (Palade et al., 1953) or the gall bladder epithelium (Yamada, 1955), which diameter was 60-80 nm. Caveolae represent an impressed design on the cytoplasmic surface as the flask shape in the ultra-cut section and the striated coat in the deeply etched image. (Rothberg et al., 1992). We have reconstructed three-dimensional images of the undercoat structure (caveolae, clathrin-coated pits, actin-based membrane skeleton) on the cytoplasmic surface of the upper cell membrane by electron tomography, with 0.85-nm-thick consecutive sections made 100 nm from the cytoplasmic surface using rapidly frozen, deeply etched, platinum-replicated plasma membranes (Morone et al., 2006). Most of caveolae or clathrin-coated pits associated with the actin-baed membrane skeleton. Here we could visualize the entire cytoplasmic surface of the plasma membrane of the fully differentiated adipocytes by the deep-etch, freeze replica electron microscopy. Most of caveolae formed over the entire surface of the cytoplasmic cell membrane, which number density was extremely high (~30 number/µm2), and were separated with the actin filament of the membrane skeleton. It found that the distribution of the caveola-based complex (so called the "caveosome") was also specific during fully differentiation to adipocytes. These caveolar formations with higher number density might play a role in the platform to function a glucose uptake on the plasma membrane of the adipocyte.

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A Functional Partnership between Intersectin-1s and Dynamin at the Neck Region of Caveolae.

I. Knezevic[1], D. N. Predescu[1], R. Neamu[2], C. Bardita[1], A. Malik[3], S. A. Predescu[1]; [1]Pharmacology, RUMC, Chicago, IL, [2]Medicine, St. Raphael Hospital, New Haven, CT, [3]Pharmacology, University of Illinois, Chicago, IL

Intersectin-1s, a multiple SH3 domain-containing protein is thought to be critically required for clathrin-coated vesicles and caveolae endocytosis due to its interactions with the proline-rich region of dynamin. Dynamin is the membrane fission GTPase shown to form a structural collar around the caveolar neck, to hydrolyze GTP and to mediate the GTP-dependent caveolae fission from the plasma membrane. The functional relevance of the interactions between intersectin-1s and dynamin remains to be established. Here, using in vivo and in vitro studies complemented by biochemical and morphological analyses of endothelial cells (ECs) expressing the SH3A domain of intersectin-1s (SH3A), we show that the binding of SH3A to dynamin stimulates the oligomerization properties of dynamin and its GTPase activity. However, dynamin assembled into rings in the presence of the SH3A is impaired in its ability to change its conformation and disassemble. This results in the accumulation of caveolae clusters and caveolae with staining-dense rings around their narrow necks, as indicated by electron microscopy morphological analysis of ECs expressing the SH3A domain. A biotin assay for caveolae internalization, applied on SH3A-transfected ECs, shows greater than 60% inhibition of biotin uptake, by reference to controls. Furthermore, cationic liposome-mediated delivery of a plasmid DNA containing the SH3A coding sequence to mouse lungs caused up to 30% decrease in transcytosis of dinitrophenylated albumin, compared to controls. These results indicate that the SH3A domain of intersectin-1s inhibits caveolae release from the plasma membrane and formation of dynamic transport vesicles, by prolonging the lifetime of assembled dynamin. Together, the findings suggest that intersectin-1s not only recruits dynamin but also regulates its activity at the neck region of caveolae.

Cell Migration I (244A-264)

244A/B188

Group Behavior and Quorum Sensing during Dictyostelium Chemotaxis.

W. Losert[1,3], C. McCann[1,2], P. Kriebel[2], C. Parent[2]; [1]Department of Physics & IPST, University of Maryland, College Park, MD, [2]Laboratory of Cellular and Molecular Biology, CCR, NCI, NIH, Bethesda, MD, [3]Institute for Research in Electronics and Applied Physics, University of Maryland, College Park, MD

The aim of our study is to quantify the requirements for signal relay during chemotaxis and elucidate the importance of environmental conditions for cell migration. Upon nutrient deprivation, the social amoeba Dictyostelium enter a developmental program allowing them to aggregate and differentiate into a multicellular organism made of spores atop a stalk of vacuolated cells. During the aggregation process individual cells sense and migrate up a chemoattractant gradient of cyclic adenosine monophosphate (cAMP), relaying the signal by synthesizing and releasing additional cAMP. This process leads to a characteristic group migration where cells align in a head-to-tail fashion and migrate in streams. We have extracted and quantified the motion of thousands of cells as they migrate toward streams, and - by labeling a fraction of cells fluorescently - the motion of cells within streams. We find that the formation of streams sensitively depends on cell plating density and volume of fluid above the cells. For instance, at a surface density of 3.5x10^4 cells/cm^2, cells stream in 50 microliters of buffer but will not stream in 600 microliters of buffer. At high cell plating densities of 7x10^4 cells/cm^2 and above cells stream with an average speed of ~10 microns/min outside steams, while at 2x10^4 cells/cm^2 cells do not stream and move with an average speed of ~5 microns/min. In addition, the fraction of cells responding to a cAMP gradient decreases with decreasing cell plating density. At very low cell plating density cells are unable to polarize and respond to a chemical signal, even close to the signal source. Together these findings suggest that quorum sensing is closely coupled to chemotaxis.

245/B189

Targeting of Calpain 1 to the Membrane by Interaction with the SH3 Motif of Cortactin Induces Integrin-dependent Migratory

Activities.

K. Bialkowska, S. Kulkarni, J. Fox; Molecular Cardiology, Cleveland Clinic, Cleveland, OH

Calpains play a critical role in migration, participating in signaling upstream of Rac in focal complexes and in extension of lamellipodia at the front of the cell as well as disruption of focal adhesion-mediated attachment at the rear. These functions are performed by calpain 1, which has been implicated in activation of integrin-mediated signaling at the front of the cell, and the highly homologous calpain 2, which has a clear role in disruption of integrin adhesion complexes at the rear. Since the calcium concentrations required to activate these proteases in vitro are higher than those thought to be attained under physiological conditions, it is assumed that factors such as phosphorylation, binding to phospholipids, or recruitment to specific sub-cellular locations regulate their in vivo functional activities. The objective of this study was to identify mechanism(s) regulating the ability of calpain 1 to initiate integrin-mediated spreading at the front of the cell. Noticing that calpain 1 contains a proline-motif (PPVP) in its N-terminal domain 1, we performed in vitro binding assays that demonstrated a specific interaction of recombinant calpain 1 but not of a proline motif calpain 1 mutant with the SH3 domain of cortactin. Endogenous calpain 1 and cortactin co-immunoprecipitated from bovine aortic endothelial cells in an adhesion-induced manner and co-localized at sites of integrin-mediated Rac activation and in integrin-induced lamellipodia. Expression of the calpain 1 mutant that failed to interact with cortactin or a fragment containing the wild-type but not the proline motif variant had no effect on the calpain 1-induced cleavage of spectrin or the integrin-mediated activation of Rac in focal complexes. However, lamellipodia extension, spreading, and migration, as well as the adhesion-induced cleavage of cortactin, were abolished. These findings provide the first demonstration that calpain 1 is recruited to the membrane by targeting to the SH3 motif of a scaffolding protein, show that its substrate specificity is determined by this proline-motif induced interaction, and identify residues on calpain 1 that mediate an interaction that is essential for integrin-dependent spreading and migration.

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The Cell Migration Molecule UNC-53/NAV-2 Is Linked to the Arp2/3 Complex by ABI-1.

K. L. Schmidt[1,2], N. Marcus[1,2], A. O. Adeleye[1], J. Webber[1], E. Stringham[1,2]; [1]Biology, Trinity Western University, Langley, BC, Canada, [2]Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada

The shape changes required for a cell or cellular process to migrate in a particular direction require a coordinated reorganization of the actin cytoskeleton. While it is known that the ARP2/3 complex nucleates actin filament assembly, exactly how the information from guidance cues is integrated to elicit ARP2/3 mediated remodeling during outgrowth is not fully understood. Previous studies have shown that C. elegans UNC-53 and its vertebrate homolog NAVs are required for the migration of cells and neuronal processes. In C. elegans, UNC-53/NAV-2 controls the longitudinal migration of the excretory canals, the sex myoblasts and the mechanosensory neurons. UNC-53 protein binds the SH2SH3 adaptor protein SEM-5/GRB2 and F-actin in vitro and UNC-53 contains several domains that suggest it participates in cytoskeletal remodeling in response to signal transduction (Stringham et al., 2002, Development). While a clear role for UNC-53 and its vertebrate homologs in cell migration has been established, little is known about how UNC-53 function is mediated. We have identified UNC-53 as a novel molecular partner of ABI-1 and found that a restricted Calponin Homology (CH) domain unique to the long-isoform of UNC-53 is sufficient to bind ABI-1 in vitro and is required in vivo for longitudinal cell migration. Analysis of abi-1(rnai) and abi-1(tm494) mutant animals reveal that ABI-1 is required for many of the same migrations as UNC-53, including the posterior migrations of the excretory canals, the anterior migrations of the PLM axon, and the dorsoventral migrations of the motorneurons. Cell-specific rescue reveals that both ABI-1 and UNC-53 function cell-autonomously in excretory cell migration, and immunofluorescence and GFP reporter analysis reveals that ABI-1 and UNC-53 are coexpressed in several cells, including motorneurons, head neurons and endocytic coelomocytes. Migration defects were also observed in RNAi of proteins known to function with abi-1 in actin dynamics in other organisms including nck-1/NCK, wve-1/WAVE and arx-2/Arp2. We propose that UNC-53, through its CH domain, may act as a scaffold to link ABI-1 to the ARP2/3 complex during actin cytoskeleton remodeling.

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Bone Morphogenetic Protein 2 Induces Pulmonary Artery Smooth Muscle Cell Migration via Recruitment of Both Wnt/β-catenin and Wnt/RhoA-Rac1 Pathways.

V. de Jesus Perez[1], T. Alastalo[2], T. Kleisli[2], J. Axelrod[3], M. Rabinovitch[2]; [1]Pulmonary and Critical Care Medicine, Stanford University, Stanford, CA, [2]Pediatrics, Stanford University, Stanford, CA, [3]Pathology, Stanford University, Stanford, CA

Background: Migration of pulmonary artery smooth muscle cells (PASMCs) to areas of ongoing vascular injury may contribute to the abnormal vascular remodeling seen in idiopathic pulmonary artery hypertension (IPAH). Work in our lab has identified that bone morphogenetic protein 2 (BMP-2) induces PASMC motility, a feature that is lost in the setting of BMP Receptor II (BMPRII) knockdown. Given that mutations in BMPRII are associated with familial and sporadic PAH, it is possible that BMP signaling may be crucial for regulation of PASMC motility. Following our recent observations that cross-talk between BMP-2 and Wnt/RhoA-Rac1 can regulate pulmonary artery endothelial cells (PAECs) motility, we proposed that a similar process could be involved in regulation of BMP-2 induced PASMC motility. Results: BMP-2 stimulated PASMC displayed RhoA and Rac1 activation after 4 hours of BMP-2 stimulation (10ng/ml). While this event coincided temporally with depression of β-catenin (βC) levels, it was preceded by an earlier rise in βC transcriptional activity within 1 hour of BMP-2 stimulation. Knockdown of βC via RNAi abrogated the BMP-2 mediated PASMC motility response suggesting that a βC gene target was critical for motility. Expression and secretion of fibronectin (FN), a known βC target, was increased after 1 hour of BMP-2 stimulation, an event that was lost in βC RNAi treated cells. Increase in FN was correlated to activation of integrin linked kinase 1 (ILK-1), an event that correlated with redistribution and colocalization of ILK-1 and disheveled Dvl), a key regulator of the Wnt/RhoA-Rac1 pathway, as evidenced by confocal and time lapse imaging. Transfection of dominant negative (DN) constructs of Dvl impaired BMP-2 induced motility supporting a critical role of Dvl in BMP-2 induced PASMC motility. Conclusions: BMP-2 promotes PASMC motility recruitment of the Wnt/RhoA-Rac1 pathway; an event that is dependent on indirect activation of ILK-1 thru the βC mediated production of FN. Abnormalities in Wnt signaling may act as a modifier in carriers of BMPRII mutations and promote development of IPAH.

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Rac'n Roll and Not Rock'n Roll : A New RhoA Pathway to Trigger Amoeboid Motility.

T. Daubon[1], L. Huyck[3,4], D. Waterschoot[3,4], A. KITZIS[1,2], C. Ampe[3,4], M. Van Troys[3,4], N. Bourmeyster[1,2]; [1]UMR6187 IPBC, CNRS, Poitiers, France, [2]LGCM, CHU de Poitiers, Poitiers, France, [3]Department of Medical Protein Research, VIB, Ghent, Belgium, [4]Department of Biochemistry, VIB, Ghent, Belgium

Bcr-Abl chimeras are produced by a reciprocal t(9;22) chromosomal translocation that fuses varying amounts of the *bcr* with the *abl* genes. The only structural difference between $p190^{bcr-abl}$, associated with acute lymphoid leukaemia, and $p210^{bcr-abl}$, responsible for chronic myelogenous leukaemia, resides in a DH/PH domain only present in $p210^{bcr-abl}$. We previously showed that Rac1 was activated in both $p190^{bcr-abl}$- and $p210^{bcr-abl}$-expressing cells whereas RhoA was activated in $p210^{bcr-abl}$-expressing cells only (Harnois et al., Oncogene, 2003). Ba/F3 cells were not spontaneously motile in 3D matrigel while Ba/F3p210 and Ba/F3p190 cells moved spontaneously. $p210^{bcr-abl}$-expressing cells presented typical **amoeboid movements** in contrast to $p190^{bcr-abl}$-expressing cells, which showed a **rolling-type mobility**. The results obtained using mutated GEF domain of Vav or $p210^{bcr-abl}$ allowed to propose a model in which Vav, in complex with Bcr-Abl, is responsible through Rac1 activation for triggering the motility of Bcr-Abl expressing cells. The GEF of $p210^{bcr-abl}$ specific of RhoA does not trigger motility by itself, but RhoA activation is crucial for committing leukaemic cells to amoeboid movements (Daubon et al., Oncogene, 2008). Bcr-Abl-expressing cells represent then a powerful model to explore the still un-understood amoeboid motility. Here, we characterized a Rac1/PAK1/LIMK1/Cofilin pathway in which each element is critical for triggering the rolling-type motility. We also showed that the classical RhoA/ROCK/MLC pathway was only partially involved in the amoeboid motility of the Ba/F3p210 cells. Surprisingly, we found that RhoA and ROCK1 induced the activation of the ADF/destrin isoform of the Actin Depolymerizing Factor family, through the specific down-regulation of LIMK2 both at the transcriptional level and by proteasome-dependent degradation. This original pathway drives to characteristic amoeboid movements. Taken together, these results on the Ba/F3 cells motility improve the understanding of the molecular mechanisms leading to these 3 dimensional motility modes.

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Synthetic Triterpenoids Target Members of the Polarity Complex and Inhibit Cell Migration.

C. To[1], J. Di Guglielmo[1], M. B. Sporn[2]; [1]Physiology and Pharmacology, University of Western Ontario, Toronto, ON, Canada, [2]Pharmacology, Dartmouth College, Dartmouth Medical School, Hanover, NH

Synthetic triterpenoids are anti-tumor agents that target various cellular functions including apoptosis, growth inhibition, anti-inflammation and cytoprotection. Previously, we have shown that the imidazolide derivative of 2-cyano-3, 12-dioxooleana-1, 9-dien-28-oic acid (CDDO-Im) can inhibit cell polarity and migration (To, C., Kulkarni, S., Pawson, T., Honda, T., Gribble, G.W., Sporn, M.B., Wrana, J.L. and Di Guglielmo, G.M., 2008, J. Biol. Chem., 283: 11700-11713). Here, we demonstrate the inhibition of cell polarity and migration extends to other synthetic triterpenoids, including the methyl ester derivative, CDDO-Me. In order to elucidate the molecular mechanisms involved, co-precipitation studies and protein array chips were utilized to identify interacting partners of biotinylated CDDO-Me. Common targets including various proteins involved in cell migration such as Rho/Rac guanine exchange factors and GTPase activating proteins, IQGAP1 and the atypical PKC isoforms, PKCι and PKCζ, were identified using these two techniques. The functional consequences of these interactions are currently being investigated to provide insight on how this class of promising anti-metastatic compounds acts to inhibit cell migration.

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HIV-1 Nef Interferes with Triggered Actin Remodelling to Affect Cell Motility.

B. Stolp[1], S. Rauch[1], L. Abraham[1], R. Grosse[2], O. Fackler[1]; [1]Virologie, Universitätsklinikum Heidelberg, Heidelberg, Germany, [2]Pharmakologisches Institut, Universität Heidelberg, Heidelberg, Germany

The HIV-1 accessory protein Nef functions as a crucial factor for AIDS pathogenesis that augments viral replication in vivo. This task is accomplished by its ability to regulate multiple host cell processes involving vesicle transport and signal transduction machineries. The molecular mechanisms of these individual activities however have remained largely unclear. Nef interferes with actin dynamics and protein sorting in T-lymphocytes upon TCR engagement. To address whether alterations of actin remodeling by Nef have general effects on cell motility, we analyzed the influence of Nef on fibroblast migration. While Nef had no effect on cell polarization, steady-state cytoskeleton organization, random cell motility or activity of Cdc42 or Rac1 GTPases, Nef expression markedly inhibited actin turnover and directed cell motility in wound closure assays. These results were extended to HIV-1 target cells by analyzing human T-lymphocytes treated with SDF-1α. The results obtained closely matched those in migrating fibroblasts: Nef expression interfered with actin reorganization and membrane ruffling upon SDF-1α treatment but had no effect on unstimulated T-lymphocytes. Consequently, Nef potently interfered with T-lymphocyte chemotaxis towards SDF-1α. The use of discriminatory Nef mutants and RNAi knock down experiments revealed that all these effects are independent of cell surface levels of the CXCR4 chemokine receptor but correlate with Nef's ability to associate with activity of the cellular kinase Pak2. In line with this scenario, the Pak downstream effector cofilin accumulates in its hyperphosphorylated inactive form in Nef-expressing cells and determines their low actin turnover rates. Thus, Nef modulates actin remodeling via Pak2-cofilin to block cellular responses to external stimuli. Modulation of directed cell motility through altered actin remodeling by Nef could affect migration of HIV infected lymphocytes in vivo and thus contribute to Nef's role in AIDS pathogenesis.

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PAR-3 Regulates Directional Migration through PI3K.

N. Itoh, M. Nakayama, T. Nishimura, S. Fujisue, T. Watanabe, K. Kaibuchi; Cell Pharmacology, Graduate School of Medicine, Nagoya University, Nagoya, Japan

Cell migration is a fundamental process in all organisms that is stringently regulated during tissue development, chemotaxis and wound healing. For a cell to migrate directionally, it must have "front-rear" polarity typically with a front leading edge and rear tail (Ridley et al., Science 2003). The activities of Rac1/Cdc42 and PIP3 levels are higher near the leading edge of migrating fibroblasts and neutrophils,

whereas RhoA activity is higher in the rear regions. Activated Cdc42 binds to the Par protein complex (PAR-3/PAR-6/aPKC) through the interaction with PAR-6. We previously found that PAR-3 directly interacts with Tiam1/STEF, Rac1-specific guanine nucleotide exchange factors, and forms a complex with aPKC-PAR-6-Cdc42●GTP, leading to Rac1 activation in the front (Nishimura et al., Nat Cell Biol 2005). We also found that Rho-kinase, an effector of RhoA, phosphorylates PAR-3 in the rear and counteracts the PAR protein complex-induced Rac1 activation, which is essential for the front-rear polarity (Nakayama et al., Dev Cell 2008). Thus, signaling cross talks among the Rho family GTPases (RhoA/Rac1/Cdc42), the PAR protein complex and PI3K play critical roles in establishing and maintaining the front-rear polarity. However, the relation between the PAR protein complex and PI3K remains largely unknown. We here found that PAR-3 formed the complex with PI3K through the direct interaction with p110, the catalytic subunit of PI3K. Knockdown of PAR-3 and the expression of the dominant negative form of PAR-3 inhibited the adhesion-induced Akt phosphorylation, suggesting that the interaction of PAR-3 with PI3K is required for the activation of PI3K. We will show the roles of the interplay between PAR-3 and PI3K in directional cell migration.

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Spatial Regulation of Phosphoinositide Turnover in Migrating MDCK Cells: Quantification by FRET Imaging.

T. Nishioka[1], K. Aoki[1], K. Hikake[1], H. Yoshizaki[3], E. Kiyokawa[2], M. Matsuda[1,2]; [1]Lab. Bioimaging and Cell Signaling, Kyoto University, Kyoto, Japan, [2]Dept. Pathology and Biology of Diseases, Kyoto University, Kyoto, Japan, [3]Dept. Biomedical Sciences, Ritsumeikan University, Kusatsu, Japan

Phosphoinositides (PtdInss) play key roles in cell polarity formation and migration. We developed a series of biosensors for PtdInss and diacylglycerol (DAG) based on the principle of Förster resonance energy transfer (FRET) and examined their distributions and metabolism at the plasma membrane. In migrating MDCK cells, the concentrations of phosphatidylinositol (4,5) bisphosphate, phosphatidylinositol (3,4,5) trisphosphate (PIP3), phosphatidylinositol (3,4) bisphosphate, and DAG were higher at the plasma membrane in the front of the cell than at the plasma membrane of the rear of the cell. The difference in the concentrations of PtdInss was estimated to be less than two-fold between the front and rear of the migrating MDCK cells. To decode the spatial activities of PtdIns metabolic enzymes from the obtained concentration maps of PtdInss, we developed a one-dimensional reaction diffusion model of PtdIns metabolism. In this model, the activities of phosphatidylinositol monophosphate 5-kinase, phosphatidylinositol 3-kinase, phospholipase C, and PIP3-5 phosphatase were higher at the plasma membrane of the front than at the plasma membrane of the rear of the cell. This result suggests that PtdInss are more rapidly turned over at the front than rear of the migrating MDCK cells.

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MRCK Drives Apical Constriction in C. elegans Gastrulation by Activating Myosin.

D. Marston[1,2], B. Goldstein[2]; [1]Dept of Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]Dept of Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC

Directed cell movements drive morphogenetic rearrangements during development. We have been developing *C. elegans* gastrulation as a new model to understand mechanisms of morphogenesis, combining cell biological, genetic, and embryological techniques. A central aim is to understand how actin networks and associated myosin motors are regulated by intra- and intercellular signaling networks. *C. elegans* gastrulation is a simple system that begins with two cells, the endodermal precursor cells, moving from the surface of the embryo to the interior, driven at least in part by constricting their apical surfaces via actomyosin contraction. We and others have shown that signals from the PAR polarity proteins and a Wnt/Frizzled pathway are required for activation of non-muscle myosin II at the apical surface of the ingressing cells. Myosin II is typically regulated through phosphorylation of its regulatory light chain by several kinases including the Rho-GTPase-family regulated ROCK (Rho kinase) and MRCK (myotonic dystrophy kinase-related CDC42-binding protein kinase). We have found that both MRCK and ROCK are required for *C. elegans* gastrulation movements, and that they have different roles. Depleting ROCK activity resulted in weak effects on gastrulation, and we have identified a specific RhoGEF and a RhoGAP with similar phenotypes, but ROCK activity does not appear to affect regulatory light chain phosphorylation. In contrast, depleting *C. elegans* MRCK caused a strong, highly penetrant defect in ingression of the endoderm cells. In embryos depleted of MRCK, endodermal cells were specified normally and had normal apico-basal distribution of PAR proteins. However, there was a dramatic reduction in the level of phosphorylated apical myosin. This myosin defect was similar to that seen when CDC-42 signaling was disrupted, potentially linking *C. elegans* MRCK to PAR mediated polarity. The gastrulation defect was partially relieved by targeting myosin phosphatase, further suggesting a role for *C. elegans* MRCK in myosin activation. These results suggest an in vivo, developmental role for MRCK, that of activating apical myosin, driving apical constriction and morphogenetic movements.

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Protrusion Rate of Migrating Epithelial Cells Is Regulated by the Organization of Contractile Actomyosin Structures in the Lamella.

J. Lim[1], M. Sabouri-Ghomi[1], C. M. Waterman[2], G. Danuser[1]; [1]Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

Directed cell migration requires continuous cycles of protrusion of the leading edge and contraction to pull up the cell rear. How these spatially distributed processes are coordinated to maintain a state of persistent protrusion remains unknown. During wound healing responses of epithelial sheets, cells along the wound-edge display two distinct morphologies: 'Leader cells' exhibit persistent edge protrusions, while the greater majority of 'follower cells' cycle between phases in protrusion and retraction. To investigate the underlying cytoskeleton dynamics, we used quantitative Fluorescent Speckle Microscopy (qFSM) to examine rates of F-actin assembly and flow relative to the local protrusion and retraction dynamics of the leading edge. Persistently protruding cells are characterized by contractile actomyosin structures that align with the direction of migration, with converging F-actin flows interpenetrating over a wide band in the lamella. Conversely, non-persistent protruders have their actomyosin structures aligned perpendicular to the axis of migration, and are characterized by prominent F-actin retrograde flows that end into transverse arcs. Analysis of F-actin kinetics in the lamellipodia showed that leader cells have threefold higher assembly rates when compared to followers. To further investigate a putative relationship between actomyosin contraction and F-actin assembly, myosin II was inhibited by blebbistatin. Treated cells at the wound edge adopted a homogeneously persistent protrusion behavior,

with rates matching those of leader cells. Surprisingly, we found that disintegration of actomyosin structures led to a significant decrease in F-actin assembly. Our data suggests that persistent protrusion in these cells is achieved by a reduction in overall F-actin retrograde flow, with lower assembly rates now sufficient to propel forward the leading edge. Based on our data we propose that differences in the protrusion persistence of leaders and followers originate in the distinct actomyosin contraction modules that differentially regulate leading edge protrusion-promoting F-actin assembly, and retraction-promoting retrograde flow.

256/B200

Differential Roles of Non-Muscle Myosin II Isoforms A and B during Fibroblast Migration in Three-dimensional Collagen Gels.

M. F. Santos[1,2], K. Matsumoto[2], K. Yamada[2]; [1]Cell and Developmental Biology, University of São Paulo, São Paulo, Brazil, [2]Laboratory of Cell and Developmental Biology, NIDCR/NIH, Bethesda, MD

Although cell migration has been characterized extensively on two-dimensional (2D) substrates, the mechanisms involved in cell migration in three-dimensional (3D) environments are poorly understood. Our objective was to determine the roles of myosin IIA and IIB isoforms during migration of human foreskin fibroblasts (HFFs) in 3D collagen gels, using two different concentrations of pepsin-digested type I collagen: 1.7 mg/ml (soft gels) and 5.0 mg/ml (dense gels). On 2D substrates, myosin IIA knockdown is known to increase HFF velocity and decrease directionality through a mechanism involving Rac1 activation, kinesin Eg5, and microtubule stabilization (Even-Ram et al., 2007). In this study, we found that cells migrated with higher velocity in soft collagen gels compared to dense gels, with no differences in directional persistence. The extent of matrix contraction by cells was much greater in soft gels, but only the cells in dense gels required proteases to migrate. Inhibition of all myosin II isoforms using blebbistatin or dual myosin IIA and IIB knockdown by siRNA reduced migration velocity by 40-60%, with no effects on cell directionality. Similar inhibition was observed after knockdown of myosin IIA alone using siRNA (~40% inhibition of velocity), whereas knockdown of myosin IIB did not affect velocity. Inhibition of Rho-kinase, a potential upstream regulator of myosin II, also decreased cell velocity, especially in soft gels. After myosin IIA knockdown, cells showed exaggerated branching processes at the front, which were not affected by inhibition of Rac1 or kinesin Eg5. EHNA, an inhibitor of the microtubule motor protein dynein, or dynein knockdown by siRNA strongly inhibited the migration of blebbistatin-treated cells, affecting both velocity and directionality. Similar results were obtained with siRNA for the GTPase Cdc42, a potential dynein regulator. In conclusion, our results suggest that myosin IIA contractility is important but not completely essential for the migration of HFFs in collagen gels, which also requires Cdc42 and dynein. Financial support: NIDCR/NIH, CAPES, FAPESP.

257/B201

The Actomyosin Linkage and Integrin-Ligand Interaction Regulate Adhesion Dynamics and Cell Migration.

L. Chen, M. Vicente-Manzanares, A. Horwitz; Cell Biology, UVA, Charlottesville, VA

Most cells show different adhesion and migration patterns when engaging different integrins or substrate ligands. We tested the hypothesis that these alterations arise from differences in myosin II (MII) activation or isoform composition. The ratios of the different MII isoforms and the level of phosphorylated MLC (pMLC), for example, can regulate adhesion maturation and turnover, protrusion rates, and polarity. CHO cells expressing α6β1 integrin migrate and protrude faster on laminin than do CHO cells expressing α5β1 migrating on fibronectin. However, the levels of pMLC are comparable in cells migrating on both substrates. Furthermore, increasing the level of MIIA or MIIB in the α6β1 cells did not have an effect on their migration, adhesion size, and protrusion rates, whereas it inhibited protrusion and increased adhesion size in cells migrating on fibronectin. Interestingly, we observed a rapid retrograde flux of paxillin in the elongated adhesions of α6β1-expressing cells on laminin or αLβ2-expressing cells on ICAM-1, but not of α5β1-expressing cells on fibronectin. Activation of α6β1 inhibits this flux, as well as cell migration and protrusion rates. Thus, the efficiency of the linkage between actin and substrates appears to contribute to the differences in migration seen in cells migrating with different integrins.

258/B202

Myosin IIB Creates a Trailing Edge/Tail and Polarizes Migrating Cells by the Localized Formation of Stable Actomyosin Filaments and Adhesions.

M. Vicente-Manzanares, M. A. Koach, L. A. Whitmore, M. Lamers, A. Horwitz; Cell Biology, University of Virginia, Charlottesville, VA

Myosin II is emerging as a critical mediator and integrator of the component processes that drive cell migration. In a previous study (Vicente-Manzanares et al., J.Cell Biol. 2007), we showed that myosin IIA resides at the cell front near protrusions, as well as in other regions of the cells; whereas IIB resides exclusively in central regions and the cell rear. We also found that cells expressing only MIIA, i.e., in the absence of MIIB or MIIC, were not polarized. In this study, we show that myosin IIB activation by MLC creates the "tail" of a migrating cell. Using myosin IIA and IIB knockdowns, B16 melanoma cells, which are deficient in MIIB, COS7 cells, which lack MIIA, and activating mutants of MLC, we have found that MLC-dependent activation of MIIB is required to form a well-defined tail opposite the dominant protrusion. Activated MIIB produces large, stable actin filament bundles and adhesions, which locally inhibit protrusion and define the pointed morphology of the tail. MIIA, in contrast, mediates adhesion disassembly and tail retraction at the cell rear and adhesion maturation at the cell front. The differential localization of MIIA and MIIB arises from the differential dynamics of the two isoforms; MIIA forms de novo filaments and adhesion maturation away from the MIIB-enriched back to form regions that support protrusion. On the other hand, MIIB resides in large, stable filaments that define the central and rear parts of the cell. The relative positioning and dynamics of MIIA and MIIB depend on the self-assembly regions in the coiled-coil C-terminus of the isoforms. These studies clarify the role of the two myosin isoforms in the formation of the trailing edge in migrating cells, reveal an unexpected role for the non-contractile region of the isoforms in determining their localization and function, and demonstrate the role of MLC-dependent activation in generating polarity during migration.

259/B203

An Adhesion-sensitive Mechanism Switches Cell Shape and Speed Determination from Dependence on Retrograde Flow to Dependence on Actin Network Growth.

E. Barnhart[1], K. Keren[2], Z. Pincus[3], G. Allen[1], A. Mogilner[4], J. Theriot[1]; [1]Department of Biochemistry, Stanford University, Stanford, CA,

[2]Technion, Israel Institute of Technology, Haifa, Israel, [3]Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CA, [4]Department of Mathematics, UC Davis, Davis, CA

Cell migration depends on spatial and temporal integration of multiple force-generating systems, including actin polymerization, myosin contraction, and adhesion. We have found that the shape and speed of rapidly migrating fish epithelial keratocytes have a biphasic dependence on adhesion strength; keratocytes crawling on intermediate adhesion strengths are fast and fan-shaped, whereas cells on low and high adhesion strengths are slow and round. To elucidate how adhesion strength determines cell shape and speed, we used fluorescent speckle microscopy to track actin network dynamics in keratocytes migrating on substrates with different adhesion strengths. Actin polymerization at the leading edge and graded retrograde actin network flow both decreased as adhesion strength increased, indicating that on low adhesion substrates cell speed is limited by weak coupling of the actin network to the underlying substrate, whereas on high adhesion substrates cell speed is limited by the rate of actin polymerization. Inhibiting myosin contraction on low adhesion substrates abolished retrograde flow and reduced actin polymerization, and promoting myosin contraction on high adhesion substrates increased actin polymerization, indicating that the balance between myosin and adhesion regulates actin polymerization as well as retrograde flow. Moreover, we found that when keratocytes migrating on micropatterned surfaces crossed boundaries between medium and high adhesion strengths, the rate of protrusion of the leading edge decreased only after the trailing edge of the cell crossed the boundary, suggesting that the balance between myosin and adhesion modulates the rate of actin polymerization indirectly, likely by modulating either membrane tension and the force exerted on the polymerizing actin network by the cell membrane or by modulating the availability of free actin monomer. These results suggest that as adhesion strength increases, the mechanism linking myosin contraction to cell shape determination switches from being primarily dependent on retrograde actin network flow to being primarily dependent on actin network assembly.

260/B204

Collective Migration of Epithelial Cells Induced by Specific Cell-Substrate Adhesions and Remodeling of Extracellular Matrix.

H. Haga, H. Tanaka, T. Mizutani, K. Kawabata; Department of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, Japan

Collective cell migrations are essential for many physiological events, such as embryonic development, wound healing and tumor invasion. We have shown that epithelial cells (MDCK cells) migrate collectively along one direction on a collagen gel, whereas the cells move randomly on a rigid collagen-coated-glass. A particular cell named a leader appears at the edge of an epithelial colony and leads the neighboring cells named followers. We found that this role assignment between a leader and followers is essential for the collective migration. In the present study, we focused on cell-substrate adhesions of an epithelial colony migrating collectively on a collagen gel, and extracellular matrix (ECM) composition remodeled by the epithelial cells. Immunofluorescence studies showed that epithelial cells on a collagen gel decomposed the collagen fibers and secreted fibronectin and laminin. The fibronectin fibers accumulated underneath the epithelial colony, and aligned toward the direction of the collective cell migration. To examine how the epithelial cells on a collagen gel adhere to the ECM including fibronectin and laminin, we observed major components of focal adhesion proteins, such as integrin beta1, vinculin, and paxillin. We found that MDCK cells except a leader did not form focal adhesions on a collagen gel. That is, only a leader formed focal adhesions and followers did not. These results indicate that epithelial cells on a collagen gel remodel the ECM and differ cell-substrate adhesions, inducing collective cell migration.

261/B205

Functional Regulation of Tumor Metastasis Suppressor Nm23 by Post-translational Modifications.

E. Lee, J. Jeong, A. Kim, S. Kim, E. Song, K. Lee; Center for Cell Signaling and Drug Discovery Research, College of Pharmacy and Division of Life & Pharmaceutical Sciences, Ewha Womans University, Seoul, South Korea

Nucleoside diphosphate kinase (NDPK, Nm23), having mutifunctions including tumor metastasis suppressor, is a housekeeping enzyme catalyzing the transfer of terminal phosphate from nucleoside triphosphate (NTP) to nucleoside diphosphate (NDP). However, the molecular regulation of Nm23 is not clearly understood. In this study, we demonstrate that enzymatic activity of Nm23 is damaged by disulfide bond formation with oxidative stress and repaired by NADPH-thioredoxin reductase-thioredoxin system. This regulation affects the downstream Rac1 activation. To understand the molecular mechanism, we thoroughly identified the cellular modifications of Nm23 by nanoLC-ESI-q-TOF tandem MS and validated these modifications with various mutants in terms of disulfide formation, enzymatic activity and invasion assay. Stable cell lines knocked down Nm23-H1 and Nm23-H2 gene using small interfering RNA (siRNA) will also give us the clues on understanding the regulatory mechanism of Nm23-H1 in various biological processes. The results suggest that oxidicative modification not in active site are major regulation of Nm23 functions. [Supported by KOSEF NCRC for CCS & DDR. J Yoon and J Lee supported by BK21]

262/B206

Tyrosine Phosphorylation of Galectin-3 in the Calpain 4 Mediated Regulation of Traction Force.

S. Menon, V. Undyala, C. Kang, K. A. Beningo; Biological Science, Wayne State University, Detroit, MI

The Calpain family of proteases plays a central role in cell migration through the cleavage at cell-substrate adhesion sites. Two members of the family, Calpain 1 and Calpain 2 each form heterodimers with a common small regulatory subunit known as Calpain 4. We have found that Calpain 4 deficient cells are defective in the production of traction forces and in mechanosensing. Surprisingly, Calpain 1 and 2 deficient cells are defective in sensing mechanical changes in the environment but not in force production. This has led us to the novel conclusion that Calpain 4 possesses a function independent of the large catalytic domains in its ability to influence traction force. It is also known that levels of tyrosine phosphorylation are elevated within focal adhesion in response to the application of mechanical stress. Therefore, to gain insight into the mechanism of Calpain 4 mediated traction force production we have used 2-D gel electrophoresis to identify proteins whose tyrosine phosphorylation levels are altered in calpain 4 deficient cells. We have identified six proteins that were differentially phosphorylated under various Calpain deficient backgrounds. One of these, Galectin-3, has been shown to play a role in migration through alterations in focal adhesion turnover. We find reduced levels of tyrosine phosphorylation of Galectin-3 only in Calpain 4 deficient cells suggesting its

importance for the production of traction force. We have verified this phosphorylation defect through pull-down assays and are currently using siRNA and mutagenesis to determine functional overlap in the production of traction forces.

263/B207

Quantitative Analysis of the Neurite Phosphoproteomes under Growth and Retraction Conditions.

F. Yang[1], Y. Wang[2], M. Gristenko[1], R. Zhao[1], W. Wang[2], E. Livesay[1], X. Du[1], M. Monroe[1], S. Purvine[1], D. G. Camp II[1], R. Klemke[2], R. D. Smith[1]; [1]Bio Separations & Mass Spect, Pacific Northwestern National Laboratory, Richland, WA, [2]Department of Pathology and Moores Cancer Center, University of California, San Diego, La Jolla, CA

Directional neurite outgrowth in response to gradient adhesive cues can be reversed by application of a lysophosphatidic acid (LPA) gradient to the neurite, which induces growth cone collapse and neurite retraction. To understand the complex signaling mechanisms that regulate neurite growth and retraction dynamics, we purified neurites from neuroblastoma cells under these conditions and applied global phosphoproteome profiling for comparative analysis. Phosphopeptides from five individual biological replicates of each growth and retraction condition were separately enriched using immobilized metal-ion affinity chromatography (IMAC) and analyzed by nano-flow high performance liquid chromatography tandem mass spectrometry (nHPLC-MS/MS). In total, 5983 phosphorylation sites were identified, of which 501 show more than a 2-fold increase in the growth phase while 326 display more than a 2-fold increase in the retraction phase. Bioinformatics analysis of the sites of phosphorylation revealed that the canonical RAS-RAF-MEK-ERK pathway is inhibited in the retraction neurites, which was further verified by Western blot. In addition, more phosphorylated ERK substrates in growth phase neurites were found than in retraction phase by motif scan, suggesting that ERK and its downstream effectors may play a critical role in neurite dynamics. This postulation is supported by the observation that constitutive activation of MEK abrogates LPA-induced neurite retraction. Quantitative identification and bioinformatics analysis of 5983 phosphorylation sites from growth/retraction neurites, led to the discovery of a novel mechanism that regulates neurite dynamics.

264/B208

Functional Characterization of the Pseudopodial Phosphoproteome Reveals Signaling Networks and Novel Kinases Involved in Cell Migration and Cancer Cell Metastasis.

R. Klemke[1], Y. Wang[1], R. D. Smith[2], J. Jacobs[2], S. Ding[2], D. Camp[2]; [1]Pathology, UCSD, La Jolla, CA, [2]Biological Sciences, Pacific Northwest National Laboratory, Richland, WA

Directed cell migration, or chemotaxis, plays a central role during embryonic development, immune modulation, and cancer metastasis. Recent evidence indicates that chemotaxis requires spatial regulation of signals that drive morphological polarization characterized by the formation of a leading pseudopodium (or invadopodium) and a rear cell body compartment. However, the mechanism underlying signal compartmentalization and morphological polarization of migratory cells is still unclear because it has not been possible to biochemically isolate the front and back compartments for large-scale protein and signal transduction analyses. Here we use microporous filters to differentially isolate the pseudopodium and cell body compartments for large-scale (> 3000 proteins) quantitative proteomic and phosphoproteomic analyses. We found that proteins involved in the regulation of actin cytoskeleton, focal adhesion dynamics, and MAPK signaling were highly enriched and/or differentially phosphorylated in the pseudopodium, whereas proteins involved in nuclear functions and metabolism were highly enriched and/or differentially phosphorylated in the cell body. Importantly, several hypothetical phosphoproteins were also differentially distributed and phosphorylated in polarized cells, including the novel tyrosine kinase (PEAK1, pseudopodial-enriched atypical kinase one). Confocal analysis of PEAK1 subcellular localization revealed co-localization with F-actin enriched protrusive pseudopodial structures. PEAK1 is activated upon integrin and growth factor ligation and interacts with the canonical Src/p130CAS/Crk/DOCK180/Rac pathway. Functional analyses indicate that PEAK1 protein is required for cell spreading and chemokine-induced chemotaxis and cancer metastasis. Our findings provided novel insight into the spatial organization of complex signal transduction pathways that control directional cell migration and cancer cell metastasis and reveal PEAK1 has a new tyrosine kinase that regulates the cell migration and invasion machineries.

Cilia and Flagella I (265-276)

265/B209

The Radial Spokes in Chlamydomonas Flagella Suppress Abnormal Calcium-dependent Motility Induced by Strong Light.

R. Gopal, P. Yang; Biological Sciences, Marquette University, Milwaukee, WI

Motile cilia and flagella steer the movement of eukaryotic cells in the aqueous environment. Various stimuli such as light and chemical compounds alter the oscillatory beating and thus the locomotion via calcium signaling. For example, light of medium intensity will turn off one of the two flagella of the *Chlamydomonas* in a calcium-dependent manner enabling cells to swim towards light, a typical phototactic response. However, how calcium alters the motility machinery, 9+2 axoneme, remains largely unknown. Radial spokes (RS) are a part of the axonemal feed back mechanism that sequentially activates dyneins to generate synchronized beating and are implicated in calcium-signaling. One of the *Chlamydomonas* spoke proteins, RSP2 is essential for the assembly of RS and thus the beating. Intriguingly, its candidate orthologs from diverse organisms contain only the N-terminal one-fourth of the molecule and lacks the C-terminus with calcium-dependent calmodulin binding sites. We postulated that the conserved sequences were required for oscillatory beating and that the C-terminus was evolved to mediate motility specific for *Chlamydomonas*. To test this, RSP2 mutants were generated. Flagella with truncated RSP2 lacking the highly conserved N-terminal DPY-30 domain are paralyzed, particularly, failing at initiating the power stroke. Interestingly, mutants expressing only the conserved N-terminus display phototaxis and a normal helical trajectory, beat frequency and waveform in dim light like the wild type control. However, these RSP2 mutants lacking the C-terminus move locally and eventually spin when light source from the microscope gradually increases. The light-induced changes do not occur if calcium is absent in the medium. These findings reveal a novel mechanism necessary for maintaining the normal motility of the biflagellate in a constant bright environment. We postulate that the

Chlamydomonas-unique RSP2 sequence modulates the mechanical feedback of RS to temper the motility changes triggered by bright light and high calcium.

266/B210

FAP221 Is the Chlamydomonas Homologue of Pcdp1 and Is a Calcium-sensitive Calmodulin Binding Protein.

C. DiPetrillo, E. Dymek, E. F. Smith; Biological Sciences, Dartmouth College, Hanover, NH

The motility of cilia for most cell types is modulated by changes in the concentration of intra-ciliary calcium. Numerous studies have indicated that calmodulin (CaM) is a key calcium sensor associated with the axoneme and plays an important role in regulating ciliary motility. Using a CaM immunoprecipitation approach, we previously identified two distinct complexes that interact with CaM in low calcium conditions (Wargo et al., 2005; Dymek and Smith, 2007). We have now precipitated CaM from axonemal extracts prepared using high calcium buffers. These precipitates include four polypeptides that are either completely lacking from low calcium precipitates or are significantly enriched in high calcium precipitates. In addition, these four polypeptides are lacking from or are considerably reduced in immunoprecipitates of extracts isolated from the central pair mutants pf18 and pf16, but are present in extracts isolated from either the pf6 or cpc1 mutants.They also cosediment on sucrose gradients. Therefore, we predict that these polypeptides form a single complex that localizes to the C1c or C1d projections of the central apparatus. Using mass spectrometry we have identified these polypeptides as FAP54, FAP46, FAP221, and FAP74 in the Chlamydomonas flagellar proteome (Pazour et al 2005). We have also determined by gel overlay assay that FAP221 is the only protein in the complex which binds CaM and that CaM binding requires high calcium. The specific CaM binding site is contained within a 58 amino acid region of FAP221 and has been confirmed by site-directed mutagenesis. Based on sequence comparisons, FAP221 is the Chlamydomonas homologue of mammalian Pcdp1 (primary ciliary dyskinesia protein 1). Although no FAP221 mutants have been identified for Chlamydomonas, mice homozygous for a mutation in Pcdp1 suffer from primary ciliary dyskinesia and die soon after birth from severe hydrocephalus (Lee et al, 2208). These combined results indicate that FAP221 is a CaM binding protein associated with the central pair of microtubules and plays an important role in modulating ciliary motility.

267/B211

Helical Rearrangement of the Axonemal Components in Chlamydomonas flagella Coupled with Ca2+ Concentrations Revealed by X-Ray Fiber Diffractions.

H. Sakakibara[1], S. Toba[1], H. Iwamoto[2], K. Oiwa[1,3]; [1]Kobe Advanced ICT Research Center, National Institute of Information and Communications Technology, Kobe, Japan, [2]Japan Synchrotron Radiation Research Institute, Harima, Japan, [3]Graduate School of Life Science, University of Hyogo, Harima Science Park City, Japan

Ca^{2+} is an important regulator of flagellar waveforms in *Chlamydomonas*. Axonemal structural components, the radial spokes and the central-pair apparatus are predicted to be involved in the Ca^{2+}-induced waveform changes. Since the axoneme is a highly ordered and precisely assembled superstructure, to understand the mechanism of Ca^{2+}-induced waveform changes requires ultrastructural studies that provide precise coordinates of axonemal components. Here, we performed low-angle X-ray fiber diffraction studies on *Chlamydomonas* axonemes, which permit analysis in nearly physiological environments and direct analysis of structural responses to Ca^{2+}. Axonemes aligned by continuous shear flow in a flow cell were irradiated with the synchrotron radiation X-ray of 0.9Å wavelength at SPring-8, BL45XU. The diffraction patterns showed several distinct layer lines, meridional and equatorial reflections. The intensity profile of 1/24 nm^{-1} meridian reflection, which mainly comes from outer dynein arms, showed that there are layer lines; the intensity peak was not on the meridian but on ~1/160 nm^{-1} from the meridian. In the diffraction pattern of outer-dynein-arm lacking mutant (*oda1*) axonemes, the layer lines at 1/24 nm^{-1}, which arise from the radial spoke, were also observed. These suggest that the helical arrangement of outer dynein arms and radial spokes exists in *Chlamydomonas* axonemes. Comparison of the diffraction patterns of axonemes and simulated diffraction patterns of helical arrangements showed that the 3 start helix was the best matched. When Ca^{2+} concentration was changed from pCa 9 to 4, the layer lines at 1/24 nm^{-1} and 1/32 nm^{-1} changed their intensities and peak positions to those of non-helical arrangement. These changes in these layer lines induced by Ca^{2+} were also observed in both radial spoke lacking (*pf14*) and central-apparatus lacking (*pf18*) mutants. Therefore, diffraction patterns at different Ca^{2+} concentrations suggest that Ca^{2+} induced helical rearrangement of the axonemal components, which arises from the roles of axonemal components other than radial spokes and central apparatus. Although we have no direct evidence, the helical rearrangement could elicit waveform changes of an axoneme.

268/B212

Outer Arm Dynein Is Regulated by a Light Chain That Tethers One Motor Unit to Tubulin.

R. Patel-King, S. King; Department of Molecular, Microbial and Structural Biology, University of Connecticut Health Center, Farmington, CT

The light chain 1 (LC1) protein is associated with the AAA+ units of the γ heavy chain (HC) motor domain within the *Chlamydomonas* outer dynein arm. This leucine-rich repeat protein contains a C-terminal helical domain that is predicted to insert into the HC AAA+ domains. To test the role of LC1 in dynein function we have expressed, in a wildtype background, mutant myc-tagged forms in which the flexibility and/or composition of the terminal helix are altered. Most myc-tagged mutant LC1 proteins behave precisely as does the wildtype protein during extraction and purification although we did observe that enhanced flexibility of the terminal helix correlates with an increased propensity to dissociate during detergent extraction of isolated flagella. Expression of mutant LC1 results in dramatic decreases in swimming velocity but has only minor effects on beat frequency. As a consequence, for some mutations the distance traveled by the cell per beat cycle is only approximately half that of wildtype. In addition to the γ HC, blot overlay and direct binding assays indicate that LC1 associates with tubulin as part of a HC-LC-microtubule ternary complex; crosslinking experiments reveal that this interaction is not sensitive to nucleotide or Ca^{2+}. To determine whether LC1 is bound to the A or B tubule of the outer doublet microtubules, we have performed immunogold labeling of axonemes from the *oda4-s7 oda11* mutant (lacks the α HC and β HC motor domain) with a polyclonal antibody against LC1. Our preliminary data suggest that LC1 is associated with the A tubule. Thus, the γ HC appears to be tethered to the same microtubule by two sites: the N-terminal region is bound via the intermediate chain/light chain complex and the motor unit by LC1. This is consistent with the distance

between the motor unit and the A tubule measured from micrographs (~6 nm), and the axial dimensions of LC1 determined by NMR spectroscopy (2.84, 3.10 and 6.98 nm). We propose that this system may act to detect alterations in flagellar curvature and allow for the sequential activation of dyneins during flagellar beating.

269/B213

Defining a Role for Radial Spoke / CSC Interaction during Axoneme Assembly and Ciliary Motility.

E. E. Dymek, E. F. Smith; Biological Sciences, Dartmouth College, Hanover, NH

Calmodulin (CaM) localizes to the axoneme and is a key calcium sensor involved in regulating ciliary motility. We previously identified a CaM containing complex (the CSC) that is localized to the base of the spoke and involved in regulating microtubule sliding (Dymek and Smith, 2007). Using several biochemical approaches we determined that one CSC member, CaM-IP2, interacts with RSP3, an AKAP that links the spoke to the doublet microtubule. Based upon this association, we hypothesized that the interaction between CaM-IP2 and RSP3 may play a role in spoke assembly and/or modulating motility. We have now determined that anti-CaM-IP2 antibodies fail to precipitate spoke proteins from either cytoplasmic extracts or membrane plus matrix fractions. Therefore, CaM-IP2 is not associated with RSP3 during assembly. CaM-IP2 may still be required for targeting and anchoring RSP3 to the doublet microtubules. There are no Chlamydomonas CaM-IP2 mutants. Therefore, to disrupt RSP3 - CaM-IP2 interaction, we generated mutations in RSP3 and transformed pf14 cells with these mutant constructs. One construct lacking C-terminal amino acids 436-516 rescued motility; however, motility was not entirely wild-type. The cells intermittently lost coordinated propagation of bends, resulting in the two flagella beating out of synchrony and cell spinning. Western blots of flagella isolated from transformants revealed that CaM-IP2 and RSP3 were present in nearly wild-type quantities, while RSP1 appeared reduced compared to wild-type axonemes. Thin section electron microscopy revealed a reduction in the number of spokes assembled in transformants. Importantly, anti-CaM-IP2 antibodies precipitated wild-type amounts of CSC, yet, significantly reduced amounts of spokes from axonemal extracts isolated from these transformants. Sucrose gradient centrifugation of extracts revealed that the CSC and radial spokes do not co-sediment. Evidently, the interaction between CaM-IP2 and RSP3 is disrupted in these transformants. These combined results indicate that interaction of CaM-IP2 with RSP3 is important for spoke targeting / stability and potentially wild-type motility.

270/B214

The Chlamydomonas IDA6 Locus Encodes a Conserved Subunit of the Dynein Regulatory Complex.

M. E. Porter[1], E. O'Toole[2], R. Bower[1], D. Tritschler[1]; [1]Genetics, Cell Bio & Dev, University of Minnesota, Minneapolis, MN, [2]Laboratory for 3D Fine Structure, University of Colorado, Boulder, CO

The dynein regulatory complex (DRC) is a group of axonemal polypeptides thought to mediate signals between the radial spokes, nexin linkages, and dynein arms (Gardner et al., 1994; Piperno et al., 1994; Nicastro et al., 2006). Several motility mutants lacking DRC subunits are associated with defects in a structure located at the junction between the inner dynein arms, radial spokes, and nexin link (Mastronarde et al., 1992; Gardner et al., 1994; Rupp & Porter, 2003). To better understand the relationship between the DRC and the inner dynein arms, we have now characterized the IDA6 gene product as a potential DRC subunit. To identify IDA6, we screened the flagellar and basal body proteomes for candidates mapping near the ida6 mutation and then tested them for rescue of the ida6 motility defect. Sequence analysis of the rescuing gene confirmed the presence of a single base pair mutation in the stop codon of the ida6 mutant. The predicted amino acid sequence corresponds to an ~65 kD, highly conserved, coiled coil polypeptide with orthologues in other organisms with motile axonemes. Antibodies directed against an IDA6 fusion protein or epitope tagged constructs reveal that IDA6 is enriched in the flagellar fraction and co-extracts in high salt extracts with other DRC subunits. Interestingly however, IDA6 is missing or reduced in a subset of drc mutants that lack DRC1 and DRC2 and assemble reduced amounts of tektin. Thin section electron microscopy and computer image averaging of the 96 nm axoneme repeat confirm that ida6 axonemes have defects in the structure of the DRC and an associated dynein heavy chain. These and other observations suggest that IDA6 is a DRC component required for facilitating interactions with at least one inner arm dynein. Supported by NIH.

271/B215

Regulation of Dynein-driven Microtubule Sliding in Flagella by an Axonemal Protein Kinase CK1.

A. Gokhale, M. Wirschell, W. Sale; Cell Biology, Emory University, Atlanta, GA

Diverse experimental evidence using *Chlamydomonas* flagellar axonemes has revealed that dynein-driven microtubule sliding is regulated by phosphorylation. One of the critical kinases involved in this regulation is CK1 (Yang and Sale, 2000). Axonemal CK1 phosphorylates dynein intermediate chain IC138 resulting in inhibition of dynein-driven microtubule sliding, thereby modulating the flagellar bend. This phosphoregulatory pathway was revealed using specific paralyzed flagellar *Chlamydomonas* mutants (*pf17, pf14*) where dyneins are globally inhibited by a mechanism involving uniform CK1-mediated phosphorylation of IC138. With the help of these mutants, as well as pharmacological inhibitors, we assessed dynein activity using an *in-vitro* microtubule sliding assay developed by Okagaki and Kamiya (1986). Consistent with our hypothesis, CK1 inhibitors block IC138 phosphorylation and rescue microtubule sliding to wild-type velocity. To further confirm the role of CK1 in the control of microtubule sliding, we "selectively" solubilized CK1 from *pf17* axonemes to test the idea that removal of CK1 would restore dynein activity. As predicted, CK1 depletion restored microtubule sliding velocity, mimicking the effect of CK1 inhibitors. Moreover, phosphatase inhibitors such as microcystin-LR, blocked rescue of microtubule sliding. Additionally, recombinant CK1 can be reconstituted onto the depleted axonemes resulting in the restoration of inhibition of dynein-driven microtubule sliding in a DRB sensitive manner. Reconstitution with "kinase-dead" CK1 does not restore the inhibition of dynein-driven microtubule sliding, thus indicating that it is the kinase activity of CK1 that is responsible for dynein activity regulation. Together, the data are consistent with the model that CK1 is localized on the outer doublet microtubules in position to regulate phosphorylation and control microtubule sliding.

272/B216

Inner Arm I1 Dynein Is Necessary for Flagellar Coordination in Chlamydomonas.

D. Howard, G. J. Pluym, J. Awowale; Biology Department, University of Wisconsin-La Crosse, La Crosse, WI

Flagellar axonemes contain at least six different isoforms of inner arm dynein. Multiple lines of evidence indicate that the inner arm dyneins play critical roles in the regulation of flagellar beating. For example, in *Chlamydomonas* the inner arm I1 (f dynein) is a downstream phosphosubstrate in a pathway that controls microtubule sliding velocity. Much of the work investigating the roles of inner arm dyneins in motility has been done either with isolated axonemes or with live uniflagellate cells. In order to determine the roles that different inner arm dyneins play in controlling flagellar beating in vivo, we used high speed digital video microscopy to analyze motility in freely-swimming, biflagellate *Chlamydomonas* cells. Wild type and cells with mutations that disrupt inner arm dyneins were imaged with DIC at 1,000 frames per sec. Mutations that disrupt I1 dynein (*ida1*, *ida3*, *ida7-1*, *pf9-2*, and *bop5-1*) cause the beating of the cis and trans flagella to become uncoordinated. In wild type cells, the two flagella are synchronized in 85-95% of the beat cycles, performing the power and recovery strokes in unison. In I1 mutants, the flagella are synchronized ≤ 50% of the time. I1 mutants can be separated into two distinct asynchrony categories. In *ida1*, *ida7-1*, *pf9-2*, and *bop5-1* cells, the asynchrony results from the fact that the two flagella beat with inherently different beat frequencies - one flagellum is 12-16 Hz slower than the other. In contrast, the two flagella beat with similar frequencies in *ida3* cells, but the flagella spend most of their time ½ cycle out of phase. When one ida3 flagellum starts the power stroke, the other starts the recovery stroke. The difference in beat frequency in the first class of mutants is similar to the difference between the cis and trans flagellum when mechanical shearing was used to create uniflagellate wild type cells (Kamiya & Hasegawa, 1987. Exp. Cell Res. 173:299). The finding that *ida3* flagella are usually ½ cycle out of phase is reminiscent of the phenotype of nonphototactic *ptx1* mutants (Rüffer & Nultsch, 1998. Cell Motil. Cytosk. 41:297). Thus, I1 is a necessary component of a system that coordinates beating between the cis and trans flagellum.

273/B217

Subassemblies of Flagellar Radial Spokes.

D. Diener, J. Rosenbaum; Molec. Cell. Dev. Biology, Yale University, New Haven, CT

Radial spokes are attached to the outer doublet microtubules of the flagellar axoneme where they are involved in coordinating the activity of the dynein arms. The spokes are composed of 23 radial spoke proteins (RSP1-23) that sediment at 20S. Five RSPs (RSP1, 4, 6, 9, and 10) form the head of the spoke, which interacts with projections of the central pair complex; the remaining RSPs make up the spoke stalk. We are examining extracts from the cell body of *Chlamydomonas reinhardtii* to determine how radial spokes are assembled. Prior to entering the flagella at least 11 RSPs assemble in the cell body to form a 12S radial spoke subassembly that includes all 5 proteins of the head and 6 proteins of the stalk. In a mutant lacking RSP3, *pf14*, the 12S complex doesn't form, but RSP4, 6, 9, and 10 assemble into a subassembly of the head complex, lacking RSP1. This head subassembly further disintegrates in a second mutant lacking the head component RSP4, *pf1*. In another mutant, *pf24*, which lacks the stalk protein RSP2, the head proteins detach from the remaining proteins of the stalk and, as in *pf1*, RSP1 separates from the remaining head proteins. In this mutant RSP3 forms a core subassembly of the stalk that lacks RSP2 and RSP5. When cytoplasmic extracts of *pf24* and *pf1* are mixed, RSP1 assembles with the other head proteins, and along with RSP2 and RSP5, joins the core stalk complex to form the 12S complex. Identifying the components of the various subassemblies of the radial spoke in these mutants will help elucidate the interactions between spoke proteins and how radial spokes are built. Supported by NIH Grant GM14642.

274/B218

The Twin Homologues in the Radial Spokehead Are Required for the Synchrony of Flagellar Beating.

M. Wei[1], P. Sivadas[1], P. Yang[1], D. Mitchell[2]; [1]Biology, Marquette University, Milwaukee, WI, [2]Cell Developmental Biology, SUNY Upstate Medical University, Syracuse, NY

The central pair apparatus (CP) and radial spokes (RS) are signature structural complexes in the 9+2 flagella. Electron microscopy showed that CP and RS contact directly and RS tilt only at the bend of beating cilia, suggesting that the precise cycle of intermittent interactions is the main mechanism converting the dynein-driven interdoublet sliding into local bending. Failures in the direct interaction or in the coupling between CP, RS and outer doublets results in paralyses or irregular stalling respectively, indicating that the CP/RS system coordinates the activation of dynein motors to generate synchronized beating. However the importance of the precision and the molecular mechanism remain unclear. Recently, we discovered two Chlamydomonas mutants defective in the gene encoding one of the two homologues, RSP4 and RSP6, in the spokehead. Contrary to the paralyzed RSP4 minus flagella that lack the entire spokehead, RSP6 minus flagella contain the other spokehead proteins and are actually motile. However, unlike the rescued control cells containing 100% wild-type like swimmers, the RSP6 minus cells become paralyzed easily and the percentage of swimmers is influenced by the medium conditions and the aeration level. The RSP6 minus swimmers are phototactic and only slightly slower than the control. However, video microscopy shows that the cells beat with normal waveform but can not maintain the helical trajectory and the two flagella lose synchrony frequently. Interestingly, more swimmers are found in the reactivated cell models than live cells, indicating the components in the membrane matrix are inhibitory. We postulate that the homologous RSP4 and RSP6 guide the precise tilt-return cycles of RS against CP to generate synchronized powerful beat at high frequency; lacking one of them result in asynchrony to be restored by beat re-initiation only possible in optimal conditions.

275/B219

Regulated Association of CrLis1 with the Chlamydomonas Outer Dynein Arm.

P. Rompolas, S. King; MMSB, University of Connecticut Health Center, Farmington, CT

The *LIS1/NUDF* gene has been implicated in the regulation of cytoplasmic dynein in various organisms, from yeasts to mammals. In humans *LIS1* mutations cause Type I Lissencephaly, a neurodevelopmental disorder which involves alterations in neuronal migration as a result of cytoplasmic dynein misregulation. However, the molecular mechanisms by which *LIS1* regulates the dynein motor are still not well understood. Previously we showed that Lis1 is also present in motile mammalian cilia and that a Lis1 homolog (CrLis1) requires the α heavy chain of the outer dynein arm motor for localization in the *Chlamydomonas* flagellum. There are two different pools of CrLis1 in the flagellum; one that is in complex with the outer dynein arm and a soluble form that is present in the flagellar matrix. We have now found that

CrLis1 is highly enriched (5-10 fold) in the flagella of cells that lack components of the radial spokes or the central pair microtubule (MT) complex; flagellar levels of CrLis1 are also increased in mutants missing parts of the inner dynein arm system. Conversely, CrLis1 is completely absent from the flagella of double mutants that lack the outer dynein arm in addition to the radial spokes or the central pair MTs. These data imply that the association of CrLis1 with the outer arm is regulated by other axonemal subsystems. We propose a model for CrLis1-outer arm interactions in which they are trafficked together into the flagellum. Following assembly of the outer arm into the axonemal superstructure, CrLis1 is released in response to signals emanating from the central pair MTs/radial spokes and is then recycled back to the cell body. Preliminary immunofluorescence experiments reveal an increased CrLis1 signal at the peri-basal body region of wild type cells but enhanced labeling of flagella in the *pf14* mutant which is in agreement with the proposed model.

276/B220

The Gene for the Axonemal PP2A B-subunit Is Defective in the Chlamydomonas Paralyzed Flagellar Mutant pf4.

C. Elam[1], L. A. Fox-Goharioon[1], M. Wirschell[1], S. Dutcher[2], W. Sale[1]; [1]Cell Biology, Emory University, Atlanta, GA, [2]Genetics, Washington University, St. Louis, MO

Diverse functional and biochemical studies, using isolated axonemes from wild-type and mutant *Chlamydomonas* cells, revealed that the PP2A A and C- subunits are anchored and localized to the outer doublet microtubules (Yang et al., 2000). We postulated that the axoneme must also contain a PP2A regulatory B-type subunit, responsible for localizing the heterotrimeric PP2A holoenzyme. To test this, we identified an axonemal PP2A B-subunit in the KCl fraction of the *Chlamydomonas* flagellar proteome (Pazour et al., 2005). The *Chlamydomonas* PP2A B-subunit gene model, (C_180129, JGI,Version 2), predicts a protein that is homologous to other members of the PR55 family of B-subunits. Using our polyclonal antibody to the N-terminus of the B-subunit, we confirmed that the PP2A B-subunit is an axonemal protein. The gene for the B-subunit mapped near the *pf4* locus on linkage group I. The *pf4* mutant strains are often palmelliod, have variable length flagella and display a jerky, medium velocity swimming phenotype. Sequence analysis of two meiotic progeny confirmed that *pf4* is defective in the gene for the PP2A B-subunit. The gene for the PP2A B-subunit in *pf4* has a base pair deletion / substitution that is predicted to result in a premature stop codon. Based on the flagellar phenotypes of *pf4*, the results are consistent with a role for PP2A in regulation of flagellar motility. The variable length flagella of *pf4* may also indicate that the B-subunit and PP2A have roles in control of flagellar assembly.

Microtubule-Associated Proteins I (277-292)

278/B222

Analysis of the Cytoplasmic Linker Protein "CLIP-170" Binding Sites on α- and β-tubulin.

K. Gupta, A. Slabbekoorn, H. Goodson; Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN

CLIP-170 is the prototype microtubule (MT) plus-end tracking protein (+TIP) and is involved in regulating MT dynamics. The question of how CLIP-170 tracks the MT-plus ends remains poorly understood due to the lack of sufficient information on the CLIP-170-MT interaction. To address this question, we are studying the binding sites of CLIP-170 on both the α- and β-subunits of tubulin dimer to better understand its role in regulating MT dynamics. It has already been shown that like many other microtubule-associated proteins (MAPs), CLIP-170 interacts with the C-terminus of the α-tubulin subunit. However, our biochemical cross-linking and subtilisin digestion experiments reveal that CLIP-170 can bind to tubulin dimers at two distinct sites: one on the C-terminal region of α-tubulin and the other located outside the C-terminus. The second site is sensitive to salt addition, suggesting the involvement of ionic interactions. This is the first demonstration of a secondary binding site for CLIP-170 on the tubulin dimer, therefore suggesting that binding at these sites might be important in plus-end tracking behavior as well as CLIP-170 activity and regulation.

279/B223

Clip170 Regulates Neuronal Polarization by Stabilizing Microtubules in the Axonal Growth Cone.

D. Neukirchen, L. Meyn, F. Bradke; Axonal Growth and Regeneration, Max-Planck Institute of Neurobiology, Martinsried-Munich, Germany

The formation of the axon is a key event in establishing neuronal polarity. Pharmacological studies implied that an increase of actin dynamics in the axonal growth cone or an increase of microtubule stability in the axon are both used as intracellular mechanisms regulating this process. However, relatively little is known about the cytoskeletal effector molecules regulating neuronal polarization. We hypothesize that microtubule binding proteins that interact with actin filaments may function as crucial regulators of neuronal polarity, as they could mediate effects on both cytoskeletal elements. Here, we present evidence that the microtubule plus end binding molecule "Cytoplasmic linker protein 170" (Clip170) is a key regulator in neuronal polarization. Overexpressing a dominant negative form of Clip170 (DN170) as well as knocking down Clip170 in hippocampal neurons reduces axon formation. Clip170 exerts its effect on axon formation by modulating microtubule behavior, as low doses of the microtubule stabilizing drug taxol rescues axon formation in DN170-expressing neurons. Analyzing the microtubular network in growth cones' transitional zones, sites of microtubule-cortex-interactions, we found that microtubules are extremely splayed out in DN170-expressing neurons. Conversely, overexpression of the microtubule-binding domain of Clip170 (MBD170) results in neurons with bundled microtubules in the growth cones. Moreover, MBD170-transfected neurons form multiple axons. Importantly, dynamic microtubule plus ends of MBD170-transfected neurons show a shift from the shaft towards the process tips compared to control cells, whereas removing Clip170 from the microtubules abolishes such a shift. We conclude that Clip170 regulates neuronal polarization by stabilizing microtubules in the axonal growth cone.

280/B224

Multiple isoforms of EB1 in Tetrahymena thermophila.

O. A. Sergeeva, D. Wilkes, D. Asai; Department of Biology, Harvey Mudd College, Claremont, CA

Microtubules are essential for a variety of cellular processes and their precise regulation is important. End Binding Protein 1 (EB1) is a member of the +TIPs family which is made up of proteins localized to the plus ends of growing microtubules. EB1 is known to regulate microtubule dynamics by both promoting initiation and maintaining the microtubules. However, the relationship between multiple classes of microtubule structures within a cell and multiple EB1 isoforms is not clear. We have investigated EB1 functional specificity/redundancy in the ciliate *Tetrahymena thermophila*, which has more than a dozen different microtubule arrays within a single cell. The *Tetrahymena* genome contains seven EB1 and EB1-like genes (EB1s A-G) and all are expressed. The predicted proteins range from 322 to 697 residues in length and are 20-37% identical to *Arabidopsis*, *Chlamydomonas*, and human EB1s. *Tetrahymena* EB1-A (361 residues) and EB1-B (412 residues) are the most similar to EB1s from other organisms. All seven of the *Tetrahymena* EB1s contain the calponin homology (CH) domain found in EB1 proteins, but only EB1-A and EB1-B additionally contain the characteristic C-terminal EB1-like superfamily motif. To examine the involvement of EB1s in the regulation of microtubules during ciliogenesis, we measured EB1 A-E gene expression during reciliation of *Tetrahymena*. EB1s B-E were each upregulated more than two-fold (EB1-B to the highest level, 3.75-fold), while EB1-A was not induced after deciliation. The macronuclear genes for EB1-A and EB1-B were disrupted individually and in combination. Neither of the single knockdowns showed obvious phenotypic changes. However, the double knockdown cells grew poorly compared to control cells. After 10 days of growth, the control cell line grew to approximately $1x10^6$ cells/mL. Under the same conditions the double knockdown culture was only about $3x10^5$ cells/mL after 10 days. The results suggest that EB1-A and EB1-B are functionally redundant in *Tetrahymena*. Supported by a grant from the National Science Foundation.

281/B225

The Microtubule Plus End-Binding Protein EB1 Is Necessary for Muscle Cell Differentiation, Elongation, and Fusion.

T. Zhang[1], K. Zaal[1], E. Reid[1], J. Sheridan[1], A. Mehta[1], G. Gundersen[2], E. Ralston[1]; [1]light Imaging Section, NIH/NIAMS, Bethesda, MD, [2]Department of Anatomy and Cell Biology, Columbia University, New York, NY

The dramatic reorganization of microtubules during muscle differentiation is at the nexus of a complete reorganization of cellular and subcellular architectures, yet it is still poorly understood. This reorganization involves changes in microtubule stability and orientation, similar to those in migrating fibroblasts, suggesting that EB1 may play a role. Yet, only EB3 was reported to affect myoblast elongation and fusion, and neither EB1 nor EB3 appears involved in microtubule stabilization (Straube & Merdes, Curr. Biol. 2007). We now report that we find EB1 necessary for elongation and differentiation of the mouse muscle cell line C2. EB1 is present on microtubule tips in undifferentiated myoblasts. Its level remains relatively stable throughout differentiation. In myotubes, it displays a microtubule plus-end pattern that suggests bipolar organization of the cells and a more prominent cytosolic pool than in myoblasts. Overexpression of a GFP-tagged EB1 C-terminal fragment, which inhibits microtubule stabilization in fibroblasts, leads to decreased C2 fusion and abnormally short and wide myotubes, whereas full-length EB1-GFP does not. In C2 cell lines, with EB1 permanently knocked down > 70% by shRNAs, the level of glutamylated tubulin, an indicator of microtubule stabilization, is decreased proportionately. Moreover, all other indicators of differentiation are affected: levels of myogenin, cadherin and β-catenin are reduced compared to controls and the accumulation of cadherin and β-catenin at the plasma membrane, normally an early myogenic event, is largely inhibited. Elongation and fusion are inhibited completely. Restoration of EB1 level through EB1-GFP expression rescues elongation and fusion. Our study suggests that, through its influence on microtubule stabilization and possibly by associating with the catenin-cadherin complex, EB1 is necessary for muscle cell elongation and fusion. We conclude that permanently knocking down EB1 reveals it as a key player in early stages of differentiation, its actions preceding those of other EB family members.

282/B226

EB1 Is Required for Normal Protrusion and Motility in Melanoma Cells.

J. M. Schober[1,2], J. M. Cain[1], Y. A. Komarova[3,2], A. Akhmanova[4], G. G. Borisy[5]; [1]Pharmaceutical Sciences, Southern Illinois University, Edwardsville, IL, [2]Cell and Molecular Biology, Northwestern University, Chicago, IL, [3]Pharmacology, University of Illinois, Chicago, IL, [4]Cell Biology, Erasmus Medical Center, Rotterdam, Netherlands, [5]Marine Biological Laboratory, Woods Hole, MA

Cells require maintenance of a polarized cell shape for persistent cell body translocation characterized by formation of protrusive actin structures including lamellipodia and filopodia at the cell front coordinated with substrate release and retraction of the cell tail. End-binding (EB) proteins are microtubule plus-end tracking proteins that regulate microtubule dynamics, and associate with cell polarity and signaling molecules. Here we address the role of EB1 protein in cell-matrix interaction and motility in mouse melanoma cells using RNA interference. B16F1 cells were transfected with plasmids encoding both soluble GFP and a short hair-pin sequence specific for EB1 or a two-nucleotide mismatch control, and cell interaction with laminin substrate was examined. Cells were fixed at 0.5, 1.0, 2.0 or 4.0 hours and stained with phalloidin for measurement of projected cell area and roundness index, an indication of a polarized cell shape. Knock down of EB1 decreased initial rate of cell spreading and caused increased cell roundness index over the whole time course. Kymographic analysis of live cells revealed non-productive protrusion caused by EB1 knock down, while control cells formed opposed retracting and protruding edges. We examined 2-dimensional motility over 24 hours and found cells depleted of EB1 exhibited decreased displacement from origin and overall decreased velocity. To gain understanding into the cause of defective cell motility we examined immunofluorescence localization of fascin, a marker of filopodia, and Arp3, a marker of actin network protrusion. Knock down cells displayed abnormal fascin localization at the basal contact surface, cell periphery and throughout the entire z-axis, while Arp3 distribution was decreased at the cell periphery. We conclude EB1 is important for maintenance of a polarized cell shape and normal cell motility, and EB1 depletion causes hyperfilopodia formation and down regulation of lamellipodia.

283/B227

SLAIN1 and SLAIN2 Are Microtubule Plus-end Tracking Proteins That Bind to EB1 and Its Homologues.

B. van der Vaart[1], I. Grigoriev[1], S. Montenegro Gouveia[1], J. Demmers[2], N. Galjart[1], A. Akhmanova[1]; [1]Cell Biology, Erasmus MC, Rotterdam, Netherlands, [2]Proteomics Center, Erasmus MC, Rotterdam, Netherlands

Microtubules are highly dynamic cytoskeletal structures essential for processes like intracellular transport and chromosome segregation during mitosis. Microtubule plus-end tracking proteins (+TIPs) specifically bind to the plus-ends of growing microtubules thereby regulating their dynamics. One of the most conserved families of +TIPs are the End Binding proteins (EBs), which are represented in mammals by three homologues, EB1, EB2 and EB3. To identify new +TIPs, we exploited the fact that the majority of known plus end tracking factors associate directly with the EBs. We used EB1-glutathione S-transferase (GST) pull down assays combined with mass spectrometry and found most known +TIPs, such as CLIP-170, CLIP-115, dynactin, CLASP1/2, spectraplakins, melanophilin and MCAK. In addition, we have identified new EB-binding partners, SLAIN1 and its homologue SLAIN2. SLAIN1 was originally described as a novel stem cell gene, and named after an amino acid stretch in the C-terminus that reads SLAIN (Hirst et al., 2006). Both SLAINs are highly conserved in vertebrates and widely expressed. To investigate the function of endogenous SLAINs, polyclonal antibodies were raised against SLAIN2 N-terminus. The antibodies recognize both SLAINs at the microtubule plus-ends, centrosome, spindle poles and the midbody. Plus-end tracking of SLAINs is mediated by the SLAIN C-terminus and is dependent on the EBs. In addition to EBs, SLAINs also interact with other +TIPs such as CLASP1 and 2, CLIP-170 and p150Glued. The C-terminus of both SLAINs terminates with a tyrosine residue, similar to that of the EBs and α-tubulin. This C-terminal tyrosine is essential for the binding of SLAINs to the CAP-Gly domains of CLIP-170. We are currently investigating SLAIN function using RNA interference-mediated knockdown in cultured cells. Hirst CE et al., Dev Biol. 2006 293:90-103.

284/B228

Tracking of Growing Microtubule Ends by Mammalian Proteins In Vitro.

S. Montenegro Gouveia[1], L. Munteanu[2], I. Grigoriev[1], R. M. Buey[3], S. Honnappa[3], M. Dogterom[2], M. Steinmetz[3], A. Akhmanova[1]; [1]Cell Biology, Erasmus Medical Center, Rotterdam, Netherlands, [2]FOM Institute for Atomic and Molecular Physics (AMOLF), Amsterdam, Netherlands, [3]Biomolecular Research, Structural Biology, Paul Scherrer Institute (PSI), Villigen, Switzerland

Microtubule plus end tracking proteins (+TIPs) preferentially bind to the growing microtubule plus ends and regulate microtubule dynamics and microtubule interactions with various cellular structures. Recently, a microtubule plus end tracking system derived from fission yeast was reconstituted in vitro from purified components (Bieling et al., 2007). We applied the same approach to mammalian +TIPs. We show that similar to the S. pombe Mal3, the mammalian EB1 and EB3 can track growing microtubule plus and minus ends on their own, in the absence of other +TIPs. We analyzed the domains responsible for this behavior and found that a monomeric fragment of EB3 containing the calponin homology domain and the unstructured linker region is able to efficiently track growing microtubule ends in vitro. Neither the acidic tail, known to have autoinhibitory properties, nor the dimerization domain is required for specific accumulation of EB proteins at the growing microtubule ends. Previously, we have shown that the Stromal Interaction Molecule 1 (STIM1), a transmembrane protein that is essential for store-operated Ca^{2+} entry, binds to EB1 and tracks growing microtubule plus ends in cells. In order to further characterize this behavior, we used the in vitro approach. We observed efficient accumulation of the GFP-tagged cytoplasmic domain of STIM1 (GFP-STIM1-C) at the growing microtubule ends in the presence of EB1 or EB3; by itself, GFP-STIM1-C is not capable of plus end tracking. We also tested MACF2 (Microtubule Actin Crosslinking Factor 2), which is known to directly bind to EB1 and exhibit EB1 dependent plus end tracking in vivo. Indeed, we could show in vitro that a short fragment of MACF2 encompassing 43 amino acids surrounding the EB1-binding site accumulates at the growing microtubule ends in an EB1-dependent fashion. We propose that STIM1 and MACF2 "hitchhike" on the EBs to growing microtubules ends. Bieling et al. 2007. Nature. 450:1100-5.

285/B229

Evidence for Competition between Actin and Microtubule Cytoskeletons for mDia1.

F. Bartolini, G. G. Gundersen; Pathology, Columbia University, New York, NY

In migrating fibroblasts, mDia is both necessary and sufficient to stabilize a subset of microtubules (Glu MTs) oriented in the direction of migration downstream from Rho signaling (Palazzo et al, NCB; Eng et al., MBC 2006). We have recently found that mDia2's actin nucleation and elongation activity is not required for its ability to induce stable microtubules and that the mDia2 FH2 domain directly binds MTs and stabilizes them in vitro (Bartolini et al., JCB, 2008). The FH2 domain is the most conserved region among formins and is also involved in actin polymerization, raising the possibility that there is competition between the two cytoskeletal systems for mDia. mDia has a higher affinity for actin than for microtubules, suggesting that if actin and microtubules exhibit mutually exclusive binding to mDia, redistribution of mDia to microtubules would only occur if the relative affinity for actin was decreased. We tested this idea by exploring whether drugs that interfere with the actin cytoskeleton might enhance mDia activity toward microtubules. We found that both Latrunculin A and Jasplakinolide stimulated the formation of Glu MTs in serum starved NIH3T3 cells. Chemical inhibition of acto-myosin contractility or ROCK activation did not result in Glu MTs formation, suggesting that Latrunculin A or Jasplakinolide do not induce microtubule stability simply by disrupting stress fiber formation. siRNA knockdown of mDia1 prevented Glu MTs formation by Latrunculin A, whereas blocking Rho had no effect. Analyses of mDia1 localization by immunofluorescence showed that mDia1 redistributed to the microtubule network upon Latrunculin A treatment. These results support the idea that there is competition between actin and microtubules for mDia1 and that mobilization of mDia1 from the actin cytoskeleton can regulate microtubule stability. These data have implications for how actin filament arrays may influence the formation of stable microtubules in cells.

286/B230

INF1 Is a Novel Formin That Induces Acetylated Microtubule Formation.

S. F. Thurston, K. Young, K. Gauthier, C. Smallwood, S. J. Copeland, J. Copeland; Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada

Formins are a widely expressed family of cytoskeletal remodeling proteins. They are characterized by the formin homology domains FH1 and FH2, which mediate actin filament nucleation and elongation. In addition, formins coordinate microtubule organization and stability via an unknown mechanism. Recent studies have shown that some formins localize on microtubule tips and may thus link microtubules to the actin cytoskeleton directly. Inverted formin 1 (INF1) is a novel formin unique to vertebrates whose function is yet to be characterized. Using immunohistochemistry and fusion protein analysis we show that INF1 co-aligns with microtubules, with this being dependent on a distinct

microtubule binding domain at the C-terminus. INF1 expressed in fibroblast cells induced actin stress fiber formation, co-alignment of microtubules with actin filaments, and the formation of bundled, acetylated microtubules. Endogenous INF1 showed an association with acetylated microtubules, and knockdown of INF1 resulted in decreased levels of acetylated microtubules. Our data suggests a role for INF1 in microtubule modification, and in coordinating microtubule and F-actin structure. Our future objectives are to study the effects of INF1 on actin and microtubules dynamics in vitro.

287/B231

Evolution of Alternative Splicing in the MAP215/Dis1 Proteins of Vertebrates.

D. Gard, S. K. Evans, E. Moellmer, J. M. Ritchie, E. D. Sanzenbacher; Biology, University of Utah, Salt Lake City, UT

We previously identified transcripts encoding two isoforms of XMAP215, a member of the MAP215/Dis1 family of MAPs, during Xenopus development. XMAP215M and XMAP215Z differ in the presence (M) or absence (Z) of a ser/thr-rich 36 amino acid insert (ins2), encoded by a single exon. XMAP215M transcript are expressed during oogenesis and predominate through gastrulation. XMAP215Z transcripts predominate in post-gastrula embryos and adults, although low levels of XMAP215M transcript can be detected in adult tissues by RT-PCR/PCR. BLAST searches revealed that the MAP215/Dis1 genes of four model fish and all mammals lack sequences encoding ins2. However, an ins2 exon was identified in the MAP215/Dis1 gene of chickens, and nested RT-PCR/PCR revealed that ins2 is expressed in MAP215 transcripts during chicken development, suggesting that ins2 arose in a common ancestor of frogs and birds. To determine the time line of ins2 evolution, we used nested primers and sequential RT-PCR and PCR to clone and sequence cDNAs and/or genomic DNA segments spanning ins2 from the MAP215 transcripts/genes of a variety of additional vertebrate species including 6 amphibians (a gymnophionan, 4 caudates, and 2 anurans), and 5 reptiles (a turtle, a snake, 2 lizards, and alligator). Surprisingly, ins2 was also identified in transcripts from P. aethipicus (African lungfish) and P. ornatipinnus (ornate bichir). However, the ins2 exon was absent from the MAP215 genes of two chondrichthyes (Squalus acanthius and Callorhincus milii), and a BLAST search failed to identify ins2 in the MAP215 gene of the cephalochordate Branchiostoma floridae. These results suggest that (1) the exon encoding ins2 arose in the common ancestor of bony vertebrates, including actinopterygii and sarcopterygii, subsequent to their divergence from chondichthyes (chimeras, sharks, and rays), and (2) the ins2 exon was subsequently lost from MAP215 genes of teleost fish and mammals. Comparison of sixteen available ins2 sequences revealed multiple conserved serine and threonines, suggesting that phosphorylation of ins2 plays an important role in regulating MAP215 activity in vivo. Supported by grants MCB0212000 and MCB0614351 from the N.S.F. and the University of Utah.

288/B232

The Spectraplakin Short Stop and the Regulation of Actin and Microtubule Cytoskeletons.

D. A. Applewhite[1], J. Currie[1], A. D. Zadeh[2], S. Rogers[1]; [1]Biology, University of North Carolina, Chapel Hill, NC, [2]Anaethesia, Pharmacology and Therapeutics, University of British Columbia, Vancouver, BC, Canada

In many cell types, directed migration requires a high degree of spatial and temporal coordination between the actin and microtubule cytoskeletal systems. Proteins that physically crosslink actin and microtubules are thought to play a significant role in mediating this coordination. We are studying Drosophila Short stop (Shot) the sole member of the spectraplakin family in flies. Spectraplakins contain an N-terminal Calponin homology (CH) domain that binds actin, a central rod composed of plakin domains and spectrin-like repeats, and a C-terminal GAS2 domain that binds microtubules. These large, multi-domain proteins are thus are poised to coordinate actin-microtubule crosstalk. Furthermore, Shot and its mammalian homologs display characteristic microtubule plus-end tracking behavior and have been shown to interact with EB1. While genetic studies of Shot have previously revealed roles in cell-cell and cell-matrix adhesion, a detailed investigation into Shot's role in coordinating actin-microtuble interactions has yet to be fully explored. We have conducted a detailed analysis of Shot behavior in Drosophila S2 cells and performed a structure/function study to determine the contribution of each protein domain to the dynamics of the protein. We also used RNAi to examine cytoskeletal dynamics in Shot-depleted cells and found that of Shot is able to regulate the rate of microtubule polymerization in an actin-depedent manner. In order to relate Shot function to cell migration, we developed a wound-healing assay based around a novel motile Drosophila cell line that is susceptible to RNAi. Depletion of Shot in this cell line leads to a hyper- motile phenotype in scratch wound assays. We present a model for Shot activity that integrates its interesting dynamics, its role in actin-microtubule crosslinking, and its contribution to cellular adhesion within the context of cell migration.

289/B233

Microtubule-associated Proteins Shape Mechanoreceptors.

S. Bechstedt[1], T. Muller-Reichert[1], J. T. Albert[3], X. Liang[1], M. C. Göpfert[2], J. Howard[1]; [1]MPI-CBG, Dresden, Germany, [2]Molekularbiologie und Biophysik Sensorischer Systeme, Zoologisches Institut Uni Köln, Cologne, Germany, [3]DRUK Research Fellow, UCL Ear Institute, London, United Kingdom

Drosophila mechanoreceptors such as those found in campaniform sensilla in wings and halteres and in the Johnston's organ in the fly's ear have a prominent microtubule-based cytoskeleton. Both receptor types are ciliated and possess characteristic ciliary modifications: the microtubule-rich tubular body in campaniform receptors and the ciliary dilation in the Johnston's organ. These modified cilia most likely serve as the site of transduction in both mechanoreceptors. We have identified two microtubule associated proteins (DCX-EMAP, katanin-like) that are specifically expressed in these mechanoreceptors, that show a characteristic subciliary localization and that, when disrupted, lead to sensory defects and cause specific ultrastructural alterations within the modified cilia. These studies show that the microtubule cytoskeleton plays a key functional role in mechanotransduction in these classes of sensory cells.

290/B234

Identification of Microtubule Associated Proteins by Mass Spectrometry in Rat Testis and Brain.

M. Miller, S. McRee, A. Rodriguez; Environmental Toxicology, UC Davis, Davis, CA

Microtubule-associated proteins (MAPs) serve a variety of important functions in the cell including the stabilization or destabilization of microtubules. The expression of MAPs can be tissue and cell type specific suggesting that they may perform unique cellular functions. The

goal of the present study was to characterize the composition of MAP proteins in the rat testis as compared to rat brain and to identify new MAPs in the testis. MAPs were isolated from both tissues by temperature dependent cycles of polymerization-depolymerization followed by NaCl induced dissociation of MAPs from microtubules. Nanospray LC-MS/MS was utilized to identify proteins present in each sample. 1,196 non-redundant proteins were identified by this method. In the brain MAPs preparation, 25 proteins were shown to have at least 5 times greater abundance than in the testis as indicated by number of peptide spectra. These included MAP2 and MAP1A, both of which have been previously reported to be predominantly expressed in neuronal tissue. 11 proteins with multiple peptide spectra were identified in the testis MAPs preparation that had no or few spectra in the brain MAPs preparation. Three of the MAPs identified in the testis (CLASP2, Doublecortin and Kinesin 5A), all of which were previously thought to be brain specific, were chosen for further study. These proteins were characterized by Western blot and immunolocalized by fluorescent techniques using testis sections prepared from frozen tissues. All three proteins were found to co-localize with Sertoli cell tubulin in seminiferous tubules in different stages of spermatogenesis. This is the first report that CLASP2, Doublecortin and Kinesin 5A are associated with microtubules in rat testis. In addition, this study provides information about the relative abundance of a large number of MAPs in brain and testis.

291/B235

Proteomic Analysis of Microtubule Associated Proteins during Macrophage Activation.

P. C. Patel[1], K. H. Fisher[2,3], E. C. Yang[4], C. M. Deane[2,3], R. E. Harrison[1]; [1]Department of Biological Sciences, University of Toronto, Toronto, ON, Canada, [2]Department of Statistics, University of Oxford, Oxford, United Kingdom, [3]Oxford Doctoral Training Centres, University of Oxford, Oxford, United Kingdom, [4]Proteomics Core Facility, Sunnybrook Research Institute,University of Toronto, Toronto, ON, Canada

Microtubules (MTs) play important role in cell division, intracellular transport, cellular motility, cell signaling and maintenance of cell morphology. For some of these functions microtubule associated elements bind the microtubules, acting mainly to stabilize them against disassembly and also regulate the dynamic properties of MTs. MT associated protein (MAP) composition is tissue and cell type specific and can vary with physiological and environmental factors. In our study, we use proteomic approach which involves a MAP purification technique based on MT cosedimentation followed by liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS) to analyze the changes in MAP abundance during activation of macrophages by IFN- and LPS. Our proteomic analysis identified 457 proteins with the ability to bind MTs in macrophage. Among them, 53 proteins were down-regulated and 69 proteins were up-regulated during activation. We categorized the identified proteins into different biological processes on the basis of the Gene Ontology (GO) annotation which mainly includes cell division/growth, cell adhesion, cytoskeleton & associated proteins, MT motor and motor associated proteins, non motor MT associated proteins (structural MAPs), metabolism, protein synthesis and degradation, cell defense, transport, and signal transduction. Specifically, the up-regulated proteins were functionally grouped under cytoskeleton associated motor and non motor proteins, cell defense and metabolism proteins. Differential expression of identified proteins by LC-MS/MS was corroborated by Western blot analysis. The purity of the microtubules from macrophage cells was confirmed by transmission electron microscopy. We used a bioinformatics analysis based on binary protein interaction data to produce putative interaction networks of MAPs. We believe that liquid chromatography coupled with mass spectrometry is a powerful approach for identifying key cytoskeletal proteins in macrophage activation. Furthermore, this study provides a valuable dataset of cytoskeleton binding proteins in macrophages.

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Initial Steps Towards the Characterization of T27A3.1, the Caenorhabditis elegans Homolog of HAP1.

K. Szeniawski[1], L. Brier[1], T. Tran[1], C. Gutekunst[2], F. Norflus[1]; [1]Natural Sciences, Clayton State University, Morrow, GA, [2]Neurology, Emory University, Atlanta, GA

HAP1 (Huntington associated protein 1) is a protein that binds with the huntingtin protein, the protein mutated in Huntington's disease. The precise role of HAP1 has not been fully determined. Some potential roles for HAP1 are in neuronal differentiation and cytoskeletal and neuronal transport. HAP1 knockout mice do not survive past postnatal day 8 indicating an important developmental role for HAP1. The Caenorhabditis elegans (C. elegans) homolog for HAP1 is called T27A3.1. Transgenic worms of T27A3.1 have localized this gene to a number of organs and to a subset of chemosensory neurons in the head and tail. Recently, knockout lines of T27A3.1 in worms have been generated. Since T27A3.1 has been found in neurons, the long term objective of this study is to characterize these worms in terms of behavioral deficiencies. However, before this can be accomplished, the worms must be backcrossed to get a completely homogenous line of worms. During the process of generating these lines, other genes may have been altered. C. elegans contains both males and self-fertilizing hermaphrodites. In order to generate the homogenous lines of worms, male wildtype worms were bred with the L4 stage of the hermaphrodite T27A3.1 knockout worms. Single worm PCR was performed to determine the genetic phenotype of the resulting offspring. The first breeding resulted in only worms that were heterozygous for T27A3.1 These heterozygous T27A3.1 worms then self fertilized. The offspring were tested to find the 25% that were homozygous for the knockout of T27A3.1. This mating scheme is repeated 5 times to generate the final knockout T27A3.1 worms. Primers were designed that flanked the T27A3.1 region. Therefore, following PCR, wildtype worms produced an 1185 bp fragment while the knockout worms produced a 419 bp fragment since these worms lacked a fragment that was present in the wildtype.

Intracellular Movement (293-313)

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Multiple-Motor-based Transport.

A. Kunwar[1], A. Mogilner[2,1]; [1]Department of Neurobiology, Physiology & Behavior, University of California, Davis, Davis, CA, [2]Department of Mathematics, University of California, Davis, Davis, CA

Intra-cellular transport via processive molecular motors plays an important role in maintaining cell structure and function. In many cases, cargoes move distances longer than expected for single motors; there is significant evidence that this increased travel is in part due to multiple motors working together to move the cargoes. However, while we understand much about the function of single motors both experimentally and theoretically, our understanding of how multiple motors work together to move cargoes is less developed. We start with a Monte-Carlo model of single motor to theoretically investigate how multiple motors work together. We have investigated the effect of non-linear force-velocity curves and stochastic load sharing on multiple motor transport using stochastic model. We are particularly interested in cargo transport by a few molecular motors which is motivated by in-vivo results that only a few motors are engaged to transport cargo. Predictions for average travel distances and mean velocities obtained from stochastic model are significantly different from those predicted using steady-state model. Our theoretical study of multiple motor transport using stochastic model also shows that single-motor force-velocity curve plays an important role in determining the ensemble function when only a few motors are engaged.

294/B238

Time-resolved Analysis of Active and Passive Transport in Living Cells.

D. Arcizet, D. M. Heinrich; Lehrstuhl Raedler, Ludwig-Maximilians Universitaet, Muenchen, Germany

Living cells exhibit exceptional dynamical properties, caused by the presence of ATP-driven motion. In particular, the intracellular transport of cargos proceeds by successive phases of diffusion and active movement along microtubules by the means of dynein and kinesin motors. While passive Brownian motion allows intracellular transport of small molecules, it becomes inefficient for large proteins, vesicles and organelles on the scale of a whole cell. Thus, motor-driven movement is crucial for many physiological processes such as axonal transport in neurons. In experimental studies, tracking of single particles is increasingly used for passive in-situ microrheology. We present an automated and reliable time-resolved identification of motility state signatures of these cytoplasmic probes. Such an approach is both experimentally challenging and of fundamental importance for our understanding of intracellular transport processes. The rolling-average algorithm we developed is based on the analysis of the local MSD and directional persistence of the tracer path. It is able to reliably separate the active and passive motion of particles in cells. We analyze the particle motion in terms of a two-state motility model: this yields the distribution of active and passive state durations as well as the distribution of the state parameters, i.e. the velocity during active phases and the diffusion coefficient of the passive motion. We investigated the motion of micron-sized beads in the amoeba Dictyostelium Discoideum (DD). DD is indeed a very convenient cell model because of its cytoskeleton simplicity and of its ability to phagocytose even large objects, such as microbeads. The distribution of active life times is found to decay exponentially with a characteristic time $\tau = 0.65$ s. The velocity distribution of active events exhibits several peaks, revealing the signature of a finite number of molecular motors working collectively. In contrast, after depolymerization of the microtubule network, the analyzed paths exhibit no significant active event, proving that active states are due to tracer transport along the microtubules exclusively.

295/B239

Discovery of New Actin-dependent Motility Mechanism Involved in Viral Transport.

J. Vaughan[1], B. Brandenburg[1], X. Zhuang[3,1,2]; [1]Chemistry and Chemical Biology, Harvard, Cambridge, MA, [2]Physics, Harvard University, Cambridge, MA, [3]Howard Hughes Medical Institute, Cambridge, MA

Polio is a model viral system for the study of nonenveloped RNA viruses, such as rhino viruses (including the common cold), enteroviruses, echoviruses, and hepatoviruses (such as hepatitis A), and if left untreated can lead to paralysis or even death. Despite decades of research, the means by which polio enters the cell and is transported to the site of genome release remains poorly understood. Using single particle tracking fluorescence microscopy, we have discovered rapid actin-dependent motility of poliovirus which achieves peak speeds of 1-5 µm/s. Multicolor fluorescence imaging and drug treatment experiments show that the motion requires an intact actin cytoskeleton but not microtubules, ruling out kinesins and dyneins as giving rise to the motion. This is in contrast to other intracellular cargoes, including the iron transporting ligand transferrin and Seneca Valley Virus (a viral relative of polio identical in size), for which transport is strongly dependent on having intact microtubules. We do not observe actin polymerization effects, such as actin comet tails or formin-induced polymerization. Furthermore, the motion is often directed over distances of many micrometers, excluding the possibility that diffusion is the cause of the motion. Together, the actin dependence, microtubule independence, absence of actin polymerization effects, and occasional observation of rapid directed motion of poliovirus along actin stress fibers suggest that a myosin may be the cause of the motion, which is a surprising possibility given that the fastest reported speeds for the transport of cargo by nonmuscle mammalian myosins are less than 0.5 µm/s.

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Quantitative Imaging Assay for Large-Scale Genetic Analysis of Cargo Transport in Drosophila Segmental Nerve Axons.

G. Yang[1], G. Reis[2], L. Szpankowski[2], L. Goldstein[2], G. Danuser[1]; [1]Dept. of Cell Biology, Scripps Research Institute, La Jolla, CA, [2]HHMI, Dept. of Cellular and Molecular Medicine, University of California San Diego, La Jolla, CA

Bidirectional transport of cargoes such as membranous organelles and protein complexes is essential to the survival and functioning of neurons. Fluorescence imaging of this process reveals its remarkably complex spatial-temporal dynamics, a result of the interactions among the diverse molecules involved, including molecular motors, cargo binding receptors, and microtubule associated proteins. Computational analysis tools are an essential part of the toolbox to investigate the molecular mechanism of this process at the system-level by overcoming the complexity of experiment data. These tools also provide sensitive analysis so that less invasive perturbation to the molecular machinery can be used to probe the system. Here, we report our latest results in developing a computational assay for automated quantitative analysis of the transport of Amyloid Precursor Protein (APP) vesicles in Drosophila segmental nerve axons. First, we introduce a computational method that reliably and efficiently recovers full cargo trajectories with sub-diffraction limit spatial accuracy in a nearly automated fashion. It provides a higher than seventy percent rate of successful cargo trajectory recovery, a two-fold improvement over our previous method. Second, we present a computational method that accurately detects and characterizes the morphology of non-diffraction-limited vesicles and use it to detect fluorescently labeled mitochondria. This method, together with our methods to detect diffraction-limited particle, provides a comprehensive tool set to automatically characterize the position, intensity, and morphology of fluorescently labeled cargoes of different

kinds. Third, we use the computational assay to resolve subtle differences in phenotypes of different mutants and to identify different modes of cargo transport under various mutations of kinesin and dynein/dynactin genes. This assay paves the way for system-level quantitative analysis of the regulatory mechanisms of axonal transport.

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Controlling Bi-directional Transport of Drosophila Lipid Droplets: The Directionality Determinant Halo Is Exclusively Expressed during Net Plus-end Transport and Physically Interacts with Kinesin-1 and Dynein.

S. L. Tran[1,2], M. Welte[1]; [1]Biology, University of Rochester, Rochester, NY, [2]Molecular Cell Biology, Brandeis University, Waltham, MA

Many intracellular cargoes move bi-directionally along microtubule tracks, transported by the opposite-polarity motor proteins Kinesin-1 and Cytoplasmic Dynein. Only very few factors have been identified that control transport directionality, and the mechanisms by which these factors regulate motor activities remain poorly understood. For lipid-droplet motion in *Drosophila* embryos, the novel transacting signal Halo functions as a directionality determinant that promotes net plus-end transport. Here we provide the first functional characterization of Halo. We show that tight temporal regulation of Halo expression is required for correct developmental regulation of plus-end transport. We determine which of two previously predicted translation start sites is used *in vivo*, define an 89 aa Halo fragment sufficient to promote plus-end transport, and identify functionally essential residues. Although Halo protein is broadly distributed throughout the cytoplasm, it likely acts directly on the transport machinery: it localizes to lipid droplets and co-precipitates both Kinesin-1 and Cytoplasmic Dynein. Finally, transport is profoundly affected by the relative expression levels of Halo and the motors, suggesting that exact stochiometry is important for regulation. We propose that Halo, Kinesin-1 and Dynein exist in common protein complexes and that the amount of Halo present in those complexes modulates motor activity.

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Regulation of the CLIP-170-dependent Interaction of Membrane Organelles with the Plus Ends of Cytoplasmic Microtubules Essential for Initiation of the Minus-end Directed Transport.

A. Lomakin[1,2], I. Semenova[1], E. Nadezhdina[2], A. Akhmanova[3], V. Rodionov[1]; [1]Center for Cell Analysis and Modeling and Department of Cell Biology, University of Connecticut Health Center, Farmington, CT, [2]A.N.Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow, Russian Federation, [3]Department of Cell Biology, Erasmus Medical Center, Rotterdam, Netherlands

Cytoplasmic microtubules (MTs) continuously grow and shorten at their plus-ends. This behavior, which is known as dynamic instability, allows MTs to search the intracellular space and make contacts with the plasma membrane, kinetochores of chromosomes, or membrane organelles. We have recently found that search-and-capture is required for the initiation of minus-end directed transport of membrane organelles in Xenopus melanophores. The major function of melanophores is redistribution of thousands of membrane-bounded melanosomes, which aggregate in the cell center or redisperse throughout the cytoplasm. Dispersion involves successive transport of melanosomes along the radial MTs and randomly arranged actin filaments. For aggregation, melanosomes that are transported along actin filaments must transfer back onto MTs for the dynein-dependent movement to the MT minus ends clustered in the cell center. Engagement of melanosomes in the minus-end directed transport during aggregation involves their capturing by the growing MT plus-ends decorated with the plus-end tracking protein CLIP-170. Using live cell imaging we show that the amount of GFP-CLIP-170-labeled MT plus-ends significantly increases after stimulation of melanosome aggregation with the hormone melatonin. This effect of melatonin treatment is explained by a rise of the frequency of MT nucleation events at the centrosome, and by a change in parameters of the MT dynamic instability. Melatonin acts by reducing cytoplasmic levels of cAMP; therefore, our data indicate that cAMP signaling facilitates capture of melanosomes by MTs by increasing the number of growing MT plus-ends bearing CLIP-170.

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Mechanism of Intracellular Transport of the Nf2 Tumor Suppressor.

L. B. Benseñor[1], K. Barlan[1], S. Rice[1], R. G. Fehon[2], V. Gelfand[1]; [1]Cell and Molecular Biology, Feinberg School of Medicine, Northwestern University, Chicago, IL, [2]Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL

Merlin is encoded by the Nf2 tumor suppressor gene that tightly controls cell-growth and contact-dependent inhibition of proliferation. Mutations in the Nf2 gene are the underlying cause of Neurofibromatosis type-2 (NF2) a familial cancer syndrome characterized by the development of sporadic tumors of the nervous system. Proper targeting of merlin dictates its association with a diverse array of regulatory proteins and is key to its growth-inhibitory function but little is known about merlin's mechanism of transport. In this work we studied the microtubule-dependent transport of wild-type merlin and mutants of merlin known to cause NF2. We observed that GFP-merlin stably expressed in Drosophila S2 cells forms particles that move bi-directionally along microtubules. When the levels of kinesin-1 or dynein heavy chains were reduced by specific RNAi probes there was a marked decrease in the movement of GFP-merlin particles. Biochemical analysis showed that merlin co-immunoprecipitates with both kinesin-1 and dynein motors. Together, these results identify kinesin-1 and cytoplasmic dynein as the microtubule motors responsible for merlin intracellular transport. We found that several NF2-causing mutants of merlin that lack tumor suppressor activity were deficient in transport. Although these mutant forms were able to form particles, these particles were non-motile. Furthermore, when wild-type and mutant merlin were co-expressed in a cell, the wild-type protein became trapped in the non-motile particles suggesting that NF2-causing mutants of merlin inhibit wild-type merlin's transport. In conclusion, these data show that merlin is transported along microtubules by molecular motors and support the idea that deficiency in microtubule-dependent transport leads to abnormal distribution of the protein and ultimately to loss of tumor-suppressor activity. Supported by the American Heart Association to LB and NIH GM52111 to VG

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UNC-83 Is a Cargo Adaptor for Kinesin-1 and Dynein at the Outer Nuclear Membrane.

M. Meyerzon, H. Petersen, N. Ly, D. Starr; Molecular and Cellular Biology, University of California, Davis, Davis, CA

Targeting of microtubule motors to specific cargo is an active area of research. Nuclei migrate to specific locations of a cell during many developmental events such as fertilization, establishment of polarity, and cell division. The C. elegans KASH protein UNC-83 specifically localizes to the outer nuclear membrane where it is required for nuclear migration in embryonic hypodermal Hyp7 cells. UNC-83 and its partner SUN protein form a bridge across the nuclear envelope, connecting the cytoskeleton to the nuclear lamina. We show that UNC-83 is a nuclear-specific cargo adaptor to recruit both Kinesin-1 and Dynein to the nuclear envelope. A yeast two-hybrid screen confirmed by GST pull-downs identified the kinesin light chain KLC-2 and three dynein regulators (NudE homolog NUD-2, BicaudalD homolog BICD-1, and dynein light chain DLC-1) as UNC-83-interacting proteins. KLC-2 interacts with the kinesin heavy chain UNC-116 to form conventional kinesin. *klc-2* and *unc-116* mutant animals had a strong nuclear migration defect similar to *unc-83* mutant animals. Immunofluorescence showed that UNC-116 and KLC-2 co-localize in part with UNC-83 at the nuclear envelope. *bicd-1(RNAi)* caused a weak nuclear migration defect that was enhanced in *bicd-1(RNAi); nud-2(ok949)* animals, suggesting that BICD-1 and NUD-2 function in parallel pathways. Further experiments showed that the egalitarian homologue C10G6.1 functions in a complex with BICD-1 and DLC-1. *dlc-1(RNAi)* is lethal, but in rare escapers, weak defects in nuclear migration were observed. These data suggest a model where UNC-83 acts as the cargo-specific adaptor between the outer nuclear membrane and the microtubule motors kinesin and dynein. In this model kinesin functions as the major force generator during nuclear migration, while dynein is involved in regulation. We are testing this model by analysis of a KLC-2::KASH construct, which we predict will target to the nuclear membrane and rescue nuclear migration in unc-83 mutants. Current work includes localization of NUD-2 and BICD-1 by immunofluorescence and analysis of the microtubule cytoskeleton structure in real time during Hyp7 nuclear migration.

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The Intraflagellar Transport Protein Injection Rate Depends on Ciliar Length.

W. Ludington, B. Engel, K. A. Wemmer, W. Marshall; Cell Biology, UCSF, San Francisco, CA

BACKGROUND: We study the mechanisms and processes by which cells maintain stable size of dynamic organelles. As a specific example we investigate length control in the eukaryotic cilia of Chlamydomonas reinhardtii, a single-celled green flagellate. Cells build cilia from the distill tip, yet all of the protein precursors of the cilium are made in the cell body, thus the cell faces the task of transporting precursor proteins out to the ciliar tip for assembly. A microtubule motor-driven process known as intraflagellar transport (IFT) accomplishes this task. Previous studies indicate that cells achieve length control by maintaining a constant amount of IFT protein in the cilia. Having a constant amount of IFT protein per cilium means that a long cilium has a smaller amount of IFT protein per unit length than a short cilium. Because the amount of IFT protein per unit length determines the assembly rate, longer cilia have a lower assembly rate. Offsetting the assembly rate is a constant disassembly rate, so when the two rates are equal, the cell has a stable length cilium. METHODS: Because the question of ciliar length control is critically dependent on a constant amount of IFT protein per cilium, in this study we ask how the cell controls levels of IFT protein in the cilia using live cell video TIRF microscopy and quantitative computational image analysis of GFP-tagged IFT proteins as well as mathematical and computational modeling in order to test our hypotheses. RESULTS: We show experimentally that the IFT injection timing, IFT raft speed, and IFT raft size are length-dependent processes. To measure these properties, we introduce a computational image processing algorithm using a modified Radon transform to reliably extract injection dwell times and IFT raft speeds from very noisy data. We also propose a mathematical model consistent with our data that explains the length-dependence of IFT injection rate based on a diffusing signal produced at the ciliar tip. CONCLUSIONS: Based on our findings we believe that ciliar length control based on a constant amount of IFT protein per cilium is achieved through an injection timing apparatus in the ciliar base that operates in a length-dependent manner.

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Growth and Elongation Within and Along the Axon.

P. Lamoureux[1], S. R. Heidemann[2], N. R. Martzke[1], K. E. Miller[1]; [1]Zoology, Michigan State University, East Lansing, MI, [2]Physiology, Michigan State University, East Lansing, MI

Most work on axonal elongation has focused on the growth cone. Here, we examine elongation and growth along the axon itself in two regimes of axonal growth: via growth cone advance and by experimentally applied tension. We find that elongation along the length of the axon initially occurs by viscoelastic stretching (creep) of the axon as seen by the movement of branch points; movements of 'docked' mitochondria in the axoplasm; and movement of marker beads along the axolemma. This is initially accompanied by thinning caliber of the axonal shaft and reduced density of mitochondria along the axon shaft. Over a time scale of hours, stretching is compensated by intercalated addition of mass as seen by a recovery of mitochondrial linear density along the shaft and recovery of axonal caliber. This 'stretch and intercalation' model for growth events within the axon shaft were entirely similar for growth cone-mediated and 'towed' elongation. These results provide additional support for the hypothesis that tension is the principal stimulus for elongation of the axon per se and that for the trailing axon, the growth cone is primarily a 'tractor,' exerting tension to guide and elongate it.

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Deterministic Mechanical Model of T-Killer Cell Polarization Reproduces the Wandering of Aim between Simultaneously Engaged Targets.

M. Kim, I. Maly; Computational Biology, Univ of Pittsburgh, Pittsburgh, PA

T-killer cells of the immune system eliminate virus-infected and tumor cells through direct cell-cell interactions. Reorientation of the killing apparatus inside the T-cell to the T-cell interface with the target cell ensures specificity of the immune response. The killing apparatus is also seen oscillating next to and between the T-cell interfaces with the simultaneously engaged target cells, in one of the most striking examples of cell movements that give the microscopist an unmechanistic impression of the cell's fidgety indecision. We have constructed a three-dimensional, numerical biomechanical model of the molecular motor-driven microtubule cytoskeleton that positions the killing apparatus. The model demonstrates that the cortical pulling mechanism that is commonly hypothesized in this field is indeed capable of orienting the killing apparatus into the functional position under a range of conditions. The model also predicts experimentally testable limitations of this commonly hypothesized mechanism of T-cell polarization. Following the reorientation, the numerical solution exhibits complex,

multidirectional, multiperiodic, and sustained oscillations in the absence of any external guidance or stochasticity. These computational results demonstrate that the origin of the strikingly animate wandering of aim in T-killer cells has a purely mechanical and deterministic explanation.

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LFA-1 Stimulated T Cell Migration Is Dependent upon Integrin Trafficking and Is Regulated by Rab GTPase and PKCε Activity.

S. Ong, M. Freeley, C. Lau, A. Davies, D. Kelleher, A. Long; Trinity College of Dublin, Dublin, Ireland

For many years it has been known that endo/exocytic cycling of surface integrin is crucial in the regulation of epithelial cell adhesion, polarization and motility. It has been proposed that vesicular transport contributes to cell migration by internalizing integrins at the retracting end of the cells and later recycling back at the leading edge to provide fresh adhesion receptors allowing cells to move forward. HuT-78 T cells, when treated with Primaquine (PMQ, a drug that inhibits receptor recycling) and stimulated to migrate through the LFA-1 receptor, showed reduction in cell area, spread of cell body and a higher index of cell roundness compared to the untreated cells when analyzed by High Content Analysis (HCA) (e.g >50% reduction in cell 1/form factor for PMQ-treated cells). Immunofluorescence analysis demonstrated that LFA-1 colocalized with three different small GTPases of the Rab family that are involved in integrin trafficking, including Rab5 (early endocytic pathway), Rab4 and Rab11 (short- and long-loop recycling pathways). Transfection of cells with a dominant negative mutant of Rab 5, S34N inhibited motility of cells in a transwell migration assay. Protein Kinase Cε, previously reported to be involved in βI integrin trafficking in migrating cells also showed colocalisation with Rab5 in LFA-1-stimulated motile T cells. Expression of kinase-dead PKCε and knockdown of PKCε expression utilizing siRNA technology, inhibited T cell migration (with the inhibition proportional to protein knockdown). In summary, LFA-1-stimulated T cell migration is a process dependent on receptor recycling and Rab5 and PKCε activity. Further studies are required to determine if there is a mechanistic link between PKCε and Rab5 in regulation of this migratory process or whether these are independent regulators.

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Characterizing the Role of Rab1a In Endocytic Trafficking.

A. Mukhopadhyay, J. Murray, A. Wolkoff; Liver Research Center and Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Objective: Fluorescent Asialoorosomucoid (ASOR) labeled early and late endosomes were isolated from rat liver and sorted through a MoFlo cell sorter. Mass spectrometric determination of the protein composition of these endosomes revealed the presence of a number of Rab proteins. One of these proteins was Rab1a. A role for Rab1a in endocytosis has not been described and was the subject of the present study. Methods & Results: Co-localization of Rab1a with Texas-Red ASOR containing endocytic vesicles revealed that 52% (n=290) of the early and 37% (n=521) of late endosomes contained Rab1a. p115 has been described as the principal effector of Rab1a in ER-Golgi vesicular transport. Three channel immunofluorescence for p115, Rab1a and fluorescent early endocytic vesicles showed that 85% (n=194) of the ASOR-vesicles associated with Rab1a did not contain p115. To investigate the importance of Rab1a in endocytic trafficking in vitro, motility of ASOR endosomes on rhodamine labeled microtubules was quantitated in the presence and absence of Rab1a antibody. It was observed that 45% (n=318) of early endocytic vesicles moved along microtubules in the absence of antibody, whereas only 25% ($p<0.001$, n=303) of the vesicles were motile in the presence of antibody. In contrast, 46% (n=217) of late endosomes were motile in the presence or absence of Rab1a antibody. Conclusions and Future Work: Proteomic studies followed by immunofluorescence have revealed the previously unrecognized presence of Rab1a in early and late endosomes although Rab1a antibody affects motility of early endosomes only. This suggests different effectors or roles for Rab1a in early and late vesicles. As maturation of early endosomes into late endosomes involves changes in associated proteins and motors, co-immunoprecipitation studies may identify Rab1a effectors in these vesicles. Further definition of the role of Rab1a may also be revealed from studies of endocytic trafficking of ASOR following knockdown of endogenous Rab1a in Huh7 cells.

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Moesin Anchors Protein Kinase A on Pigment Granules to Regulate Pigment Dispersion in Melanophores.

K. Ikeda, I. Semenova, P. Ivanonv, V. Rodionov; Cell Biology, UCONN Health Center, Farmington, CT

Major signaling cascades have been shown to play a role in the regulation of intracellular transport of organelles. In Xenopus melanophores, aggregation and dispersion of pigment granules is regulated by the second messenger cAMP through the protein kinase A (PKA) signaling pathway. PKA is bound to pigment granules where it forms complexes with molecular motors involved in pigment transport. Association of PKA with pigment granules occurs via binding to A-kinase anchoring proteins (AKAPs), whose identity remains largely unknown. Here we used mass-spectrometry to investigate 80 kDa AKAP detected in preparations of purified pigment granules. We found that tryptic digests of granule protein fractions enriched in the 80 kDa AKAP contained peptides that corresponded to the actin-binding protein moesin, which has been shown to function as AKAP in mammalian cells. We also found that recombinant Xenopus moesin interacted with PKA in vitro, co-purified with pigment granules, and bound to pigment granules in cells. Overexpression in melanophores of a mutant moesin lacking conserved PKA binding domain did not affect aggregation of pigment granules, but partially inhibited their dispersion. We conclude that Xenopus moesin is AKAP whose PKA scaffolding activity plays a role in the regulation of pigment dispersion in Xenopus melanophores.

307/B251

Time-lapse Observation of Catecholamine-induced Morphological Changes of Lipid Droplets in Adipocytes.

M. Nagayama[1], K. Shimizu[2], T. Taira[2], T. Uchida[1], K. Gohara[1]; [1]Applied Physics, Grad Sch Engineering, Hokkaido Univ, Sapporo, Japan, [2]Primary Cell Co., Ltd., Ishikari, Japan

Lipid droplet (LD) is the most characteristic organelle of adipocytes in mammals. Adipocytes store excess energy as triglyceride in the LDs and release the energy as free fatty acid and glycerol from the LDs via lipolysis. To date, many molecular processes involved in the mobilization as well as accumulation of lipid have been identified using biochemical and molecular biological techniques. On the other hand,

how the molecular processes lead to spatial and temporal dynamics, such as morphological changes, of LDs in adipocytes during lipolysis have not been clarified yet. In the present study, we performed time-lapse observations of adipocyte when lipolysis was induced by stimulation with catecholamine (either norepinephrine or isoproterenol). The adipocyte used in the experiment was prepared as follows: primary stromal-vascular cells were isolated from rat mesenteric adipose tissue, and then were differentiated into adipocytes in vitro. According to the time-lapse observations, large LDs shrank rapidly from 2 to 8 hours after the stimulation and finally disappeared in some but not all of adipocytes. In other adipocytes, large LDs were preserved and newly small LDs were developed continuously in the cell periphery although lipolysis was induced. To assess translocation of endogenous lipases due to stimulation with catecholamine, we also performed immunofluorescence observations of adipose triglyceride lipase (ATGL) and phospho-hormone sensitive lipase (phospho-HSL). The catecholamine stimulation did not alter a punctate distribution of ATGL in the cytoplasm of all adipocytes. In contrast, phospho-HSL was localized exclusively on the surface of LDs in some but not all of adipocytes after the stimulation. In summary, our time-lapse observation would provide an exact description of catecholamine-induced diverse morphological changes of LDs in adipocytes.

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Actin- and Microtubule-dependent Reorganization of Golgi Complex Induced by Activation of mDia1 Formin.

Y. Zilberman, A. Bershadsky; The Weizmann Insitute of Science, Rehovot, Israel

Mammalian cells are known to concentrate elements of Golgi complex around the centrosome in a microtubule-dependent manner. The cytoskeletal mechanisms determining the Golgi complex integrity are, however, poorly understood. Here, we show that activation of RhoA or its immediate target, mammalian Diaphanous related formin 1 (mDia1) produces in HeLa cells disintegration of Golgi complex and dispersion of its elements over wide cytoplasmic area. Accordingly, depletion of the mDia1 abolished RhoA effect on Golgi. mDia1, similarly to other formins, is a potent stimulator of actin nucleation and elongation, operating via its formin homology (FH) domains FH1 and FH2. Expression of active form of mDia1, comprising the FH1 and FH2 domains, led to Golgi dispersion together with augmentation of actin bundle (stress fiber) formation. Co-expression of active mDia1 with excess of FH2 domain significantly inhibited the mDia1 effect on Golgi, while stress fiber formation was not affected. Thus, mDia1-driven Golgi dispersion is not just a result of stress fiber augmentation. At the same time, inhibition of actin polymerization by latrunculin B, or myosin-II activity by blebbistatin, abolished the mDia1-induced Golgi complex dispersion. Microtubule stabilization by taxol did not induce significant Golgi dispersion by itself, but greatly enhanced the mDia1 effect, indicating to involvement of microtubules in the mDia1-mediated Golgi dispersion. We hypothesize that the effect of mDia1 on Golgi integrity is mediated through interaction of mDia1 with dynactin complex involved in molecular motor-driven translocation of Golgi elements along microtubules. We demonstrated that mDia1 co-immunoprecipitates with actin related protein 1 (Arp1), which is an essential component of dynactin complex. Active mDia1 inhibited the movement of Arp1 patches along microtubules, and this effect, similarly to Golgi dispersion, was abolished by depolymerization of actin filaments by latrunculin B. We thus propose that mDia1 serves as a dynamic link between dynactin and the actin cytoskeleton.

309/B253

HUMMR, a New HIF-1-induced Protein, Retards Mitochondrial Transport during Hypoxia.

Y. Li[1], S. Lim[2], H. J. Federoff[2], D. Rempe[1]; [1]Center for Neural Development and Disease, University of Rochester, Rochester, NY, [2]School of Medicine, Georgetown University, Washington, DC

Mitochondrial function and transport are important for maintaining cellular function and dysfunction of mitochondrial transport is implicated in multiple neurological diseases. Hypoxia and Hypoxia Inducible Factor 1 (HIF-1) are known to influence mitochondrial function. Our objective was to examine the function of a protein that is induced by hypoxia and localizes to mitochondria. Methods: Transgenic neuronal and astrocytes cultures were employed to examine expression of our novel protein (termed HUMMR), and its dependence on HIF-1 function. Overexpression and loss of function (shRNA) was utilized to examine HUMMR function. Results: The hypoxic induction of HUMMR, and its dependence on HIF-1 function was confirmed on both transcriptional and translational level in astrocytes and neuronal cultures. Immunocytochemistry showed HUMMR is localized in mitochondria. In astroctyes, overexpressing HUMMR induced mitochondrial network collapse into peri-nuclear clusters. This phenomenon was independent of mitochondrial fusion protein mitofusin 1 and 2, and was not reversed by mitochondrial fission protein hFis and Dynamin Like Protein 1 (DLP1). Interestingly, HUMMR interacted with proteins responsible for mitochondrial transport, such as Mitochondrial Rho GTPase (Miro) and Milton. Loss of HUMMR function, achieved by RNAi, markedly increased mitochondrial motility in neurons during hypoxia. Furthermore, we demonstrated that HIF-1 affected mitochondrial movement in the same way through HUMMR. In conclusion, HUMMR (Hypoxia Upregulated Mitochondrial Movement Retardant), a novel HIF-1 target, reduces mitochondrial movement during hypoxia. Given that the oxygen tension of the brain (2-5% oxygen) is well below that in room air, these findings have broad implications for mitochondrial transport and downstream processes in both physiological and ischemic conditions.

310/B254

Role of Actomyosin-based Force Generation in Mitochondrial Movement in Drosophila Neurons.

D. Pathak, P. Hollenbeck; Biological Sciences, Purdue University, West Lafayette, IN

Tightly-regulated transport and distribution of mitochondria are critical for cellular homeostasis in large, asymmetric cells such as neurons. Kinesin-1 and cytoplasmic dynein move mitochondria on microtubules over long distances in the axon, but mitochondrial movement is saltatory, bidirectional, and employs actin as well as MT tracks. We aimed to identify the myosin motor(s) that drive actin-based mitochondrial movement, and to elucidate their role in complex transport behavior, including organelle docking. We have used RNA interference to deplete myosin motors in cultured Drosophila primary neurons expressing mitochondrially-targeted GFP (mito-GFP). We overcame the resistance of primary cultures to RNAi by employing a fly line expressing both mito-GFP and the Sid 1 RNA transporter from C. elegans. Sid 1-expressing neurons showed RNAi inhibition of protein expression 2.5-fold greater than control cells. We used dsRNA in Sid 1/mito-GFP neurons to knock down expression of myosins II, V, or VI and quantified the effects on mitochondrial axonal transport. Myosin V depletion increased the mean duty cycle for mitochondrial movement in both anterograde and retrograde directions and

particularly increased the frequency of longer runs, indicating that the normal role of myosin V is to disrupt protracted MT-based movement. Although myosin V depletion increased mitochondrial transport in both directions, the effect was more pronounced for anterograde movement, suggesting a preferential disruption of kinesin-based movement. An additional effect of myosin V knock down was the doubling of mean mitochondrial length. Myosin VI knock down produced a selective increase in retrograde duty cycle, while the anterograde duty cycle and mitochondrial length remained unaffected. Myosin II depletion gave no apparent phenotype. These data indicate that myosin V and VI play related but distinct roles in regulating MT-based mitochondrial movement, probably by disrupting long runs and facilitating organelle docking.

311/B255

The Dynamics of Mammalian P Body Transport, Assembly and Disassembly In Vivo.

A. Aizer[1], Y. Brody[1], L. Ler[2], N. Sonenberg[2], R. Singer[3], Y. Shav-Tal[1]; [1]The Mina & Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat Gan, Israel, [2]Department of Biochemistry and McGill Cancer Center, McGill University, Montreal, QC, Canada, [3]Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

The fate of exported mRNAs in eukaryotic cells is highly regulated and mRNAs are degraded by several mRNA decay mechanisms, some involving mRNA decapping. The decapping machinery is enriched in cytoplasmic foci termed processing bodies (P bodies). These contain translationally repressed mRNPs associated with translation repression or the mRNA decay machinery. We followed the dynamic properties of PBs in living human cells and measured the kinetics of PB transport and assembly using quantitative time-lapse microscopy. We asked whether PBs actively scan the cytoplasm in pursuit of mRNAs or if the biophysical mechanism of interactions is diffusion-based. Single particle tracking analysis showed that PB mobility was mostly random but confined, due to cytoskeletal anchoring, mainly to microtubules. Still, some PBs exhibited directional motion with high velocities on microtubules. We examined the fate of PB assembly and mobility during the disruption of the microtubule network, and in relation to inhibition of the transcription and translation processes. PB mobility and diffusion coefficients were compared to mitochondria, ER, peroxisomes, SMN bodies, and stress granules. We identified post-translational modifications that might regulate the interactions between PB components. Finally, using photobleaching we could identify major differences in the flux of PB components in and out of PBs. Our measurements and calculations indicate that PB complexes and their protein components are highly dynamic. However, an active cytoplasmic PB-scanning mechanism does not occur and the diffusive motion of RNAs bound to the microtubule network is sufficient for targeting into PBs. We propose that: 1) PB association with the cytoskeleton is a necessity for encounters with mRNA molecules, and 2) PB disassembly during mitosis is a means by which the cell protects mRNAs from degradation.

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Role of Cytoskeleton in the Activation and Transportation from Cytosol to Nucleus of STAT5 in MCF-7 Breast Cancer Cells.

E. Perez Salazar, M. Lopez-Perez, T. Robledo, L. A. Castro-Sanchez, A. Soto-Guzman; Cell Biology, Cinvestav-IPN, Mexico DF, Mexico

Background/Aims: Epidermal growth factor receptor (EGFR) signaling is mediated by Ras activation and also by signal transducers and activators of transcription (STAT) proteins, which following phosphotyrosine-dependent dimerization by-pass classical kinase cascades, enter the nucleus to regulate genes directly. While STAT5 activation and translocation into the nucleus have been studied, the role of cytoskeleton in the activation and transportation of STAT5 from cytosol to the nucleus, remains to be analyzed in detail. Our aim was to study the role of actin fibers and microtubule network in STAT5 phosphorylation and transportation from cytosol to the nucles in MCF-7 cells stimulated with epidermal growth factor (EGF). Methods/Results: STAT5 phosphorylation at Tyr-694 was measured by immunoprecipitation and Western-blotting. EGF promoted an increase in the phosphorylation of STAT5 at Tyr-694, as revealed by site-specific antibodies. By using nuclear extracts from cells stimulated with EGF and EMSA, we demonstrated that EGF promoted STAT5 nuclear translocation and an increased in STAT5 DNA binding activity. To investigate the participation of cytoskeleton in STAT5 activation, cells were treated with cytochalasin D or colchicine, prior to stimulation with EGF. Prevention of microtubule and microfilament polymerization induced a partial inhibition of STAT5 nuclear translocation and STAT5 DNA binding activity. However, STAT5 phosphorylation at Tyr-694 was dependent of the integrity of microtubule network and it was independent of actin cytoskeleton integrity. These results strongly suggest the participation of microtubule network and one mechanism of transportation of STAT5 from cytosol to nucleus. We evaluated by immunoprecipitation assays whether EGF induces the formation of a complex between STAT5 and tubulin protein and with kinesin family members. EGF induced the formation of the associations STAT5-tubulin and STAT5-kinesin heavy chain in a fashion dependent of cytoskeleton integrity. Conclusion: our results demonstrate that cytoskeleton plays an important role in STAT5 activation and translocation into the nucleus in MCF7 cells stimulated with EGF.

313/B257

Regulation of IL-1α Secretion by Caspase-8.

D. Lee, P. Lee, G. Chen, C. Jamora; Biology, UC San Diego, La Jolla, CA

The coordination of cell death and proliferation is the foundation of skin homeostasis and regeneration. Disruption of the signaling pathways of these two processes can result in diseases and cancer. Misregulation of apoptosis can lead to abnormal wound healing, such as hypertrophic scar, keloid formation, and several cutaneous diseases, such as psoriasis and TEN (Toxic epidermal necrolysis). We found that caspase-8, an essential mediator of the apoptotic signaling pathway, plays a non-apoptotic role in stimulating inflammation and proliferation during a wound healing response in the mammalian skin. The expression of caspase-8 is downregulated at the wound closure site and IL-1α is a key factor that orchestrates the multiple signal pathways of skin restoration. How caspase-8 induces the secretion of IL-1α is therefore fundamental in understanding the process of wound repair. However, IL-1α lacks a signal peptide for transport and it is secreted by an unconventional, endoplasmic reticulum/Golgi-independent, pathway. To elucidate how caspase-8 regulates IL-1α secretion, we examined the subcellular localization of caspase-8 in vitro. Interestingly, the immunofluorescent staining indicates that caspase-8 colocalizes with microtubule and an in vitro microtubule binding assay confirmed that caspase-8 associates with microtubules. The organization of microtubule in the caspase-8 KO keratinocytes is disrupted compared to the WT cells. Furthermore, changes in microtubule cytoskeleton network have been reported to regulate protein transport. Our results confirm that the secretion of IL-1α in keratinocytes is also mediated by

microtubule dynamics. Altogether, we found the ability of caspase-8 to regulate microtubule organization may link this enzyme to the secretion of IL-1α.

Unconventional Myosin (314-335)

314/B258

A Novel Interaction between Myosin-1d and Aspartoacylase, an Enzyme Implicated in Myelination.

A. Benesh, M. Tyska; Cell and Developmental Biology, Vanderbilt University, Nashville, TN

Myosin-1d (Myo1d) is an actin based motor protein that is widely expressed in many tissue types including the brain, intestine, and kidney. While previous studies suggest that the Drosophila Myo1d homologue is involved in body patterning during development, the cellular functions of Myo1d and the identities of its cellular cargoes remain unknown. Thus, we initiated a yeast-two-hybrid screen to find Myo1d binding partners; and using the Myo1d tail as bait, we identified aspartoacylase (ASPA). ASPA is a member of the carboxypeptidase family, which hydrolyzes the carboxyl terminus of target peptides in an array of physiological contexts. ASPA is highly expressed in the brain and deacetylates N-acetyl-L-aspartate (NAA) to synthesize acetyl groups for incorporation into fatty acids during myelination of neurons. To confirm our yeast-two-hybrid result, we performed biochemical pulldown assays, which showed that His-tagged ASPA purified from E. coli is capable of interacting with EGFP-Myo1d in cell culture lysates. In addition, EGFP-ASPA colocalizes with mCherry-Myo1d in rat C6 glioma cells, and Neuro2a cells which do not express either protein endogenously. Lastly, immunoflorescence data from mouse brain sections show Myo1d and ASPA colocalize in the same cell types in the cortex and hypothalamus. Intriguingly, linkage analysis suggests that Myo1d single nucleotide polymorphisms are associated with autism. In addition, clinical studies have revealed that NAA brain levels in patients with autism are greatly reduced, and other data has shown that individuals with autism exhibit altered brain symmetry due to changes in myelination during the first two years of life. We hypothesize that Myo1d transports ASPA to sites of myelination and that disrupting this interaction could result in myelination defects and perturbations in neurodevelopment. Our future studies will focus on the Myo1d/ASPA interaction in this neurobiological context.

315/B259

Class I Myosins Regulate Plasma Membrane Tension in Polarized and Non-polarized Cell Types.

R. Nambiar, M. Tyska, R. McConnell; Cell and Developmental Biology, Vanderbilt University, Nashville, TN

Plasma membrane tension is known to be regulated by the level of "adhesion energy" available for linking the actin cytoskeleton and plasma membrane, yet the molecules that contribute to adhesion energy are unknown. An example of elaborate cell morphology, which highlights the importance of membrane-cytoskeleton adhesion is provided by the brush border (BB) found on the apex of intestinal epithelial cells (IEC). The BB is an array of cylindrical membrane protrusions that extend away from the cell surface, each one supported by a parallel bundle of actin filaments. To stabilize this convoluted morphology, the IEC must furnish the BB with high levels of membrane-cytoskeleton adhesion. One candidate molecule for this task is myosin-1a (Myo1a), a membrane-binding motor, which is present at high levels in the microvillus. To determine if Myo1a plays a role in regulating membrane tension, we developed an optical tweezer assay to probe membrane mechanics in isolated organelles and live cells. Using this assay, we demonstrate that the slope of the membrane force-extension curve is drastically reduced in BBs isolated from Myo1a knockout mice relative to WT controls (i.e. less force is required to extend the membrane a given length). We also show in polarized epithelial cells, that over-expression of full length Myo1a caused membrane tension to increase, whereas expression of dominant negative (DN) Myo1a dramatically reduced tension. Intriguingly, we observed that DN expression significantly reduced the cell's ability to stabilize the local formation of multiple tethers, further suggesting that the mean contact density between cytoskeleton and membrane is reduced in the presence of this non-functional Myo1a fragment. To determine if these effects were specific to Myo1a and/or dependent on the presence of microvilli, we carried out similar studies in NIH-3T3 cells. Although these cells lack microvilli, the over-expression of five different class I myosins resulted in an increase of the average number of tethers pulled relative to controls. These studies are the first to show that class I myosins play a direct role in regulating membrane tension through their contribution to membrane-cytoskeleton adhesion.

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Structural and Compositional Changes in the Enterocyte Due to Loss of Myosin-1a and Villin Lead to Anoikis.

A. T. Mackey[1], C. L. Chow[1], S. Robine[4], D. Louvard[4], M. Mooseker[1,2,3]; [1]Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT, [2]Cell Biology, Yale University, New Haven, CT, [3]Pathology, Yale University, New Haven, CT, [4]UMR 144 Centre National de la Recherche Scientifique, Institut Curie, Paris, France

Myosin-1a (Myo1a) and villin are major Ca2+ regulated actin-binding proteins of the enterocyte brush border (BB) cytoskeleton. To assess the potential functional synergy between Myo1a and villin, a mouse null for both Myo1a and villin was generated and characterized. While no overt phenotype was observed on the whole animal level, a series of unique compositional and structural defects within the enterocytes of the double knock out (KO) were observed. Defects within the BB itself were a recruitment of myo1e and γ-actin in the microvilli (MV), phosphorylation of ezrin within the MV, and an increase in tropomyosin (TM), including infiltration of TM into the MV. Within the terminal web (TW), increased levels of myosin light chain kinase within the M/V KO enterocyte lead to increased phosphorylation of myosin light chain and, presumably, increased myo2a activity. Additionally, there is an increase in caldesmon and phosporylated caldesmon in the M/V KO which could also influence myo2a activity in the TW. The M/V KO enterocyte also exhibits aberrant ER and endosome organization based on calreticulin and EEA1 staining. Consistent with the immunostaining, TEM analysis of the M/V KO enterocyte reveals increased sub-apical vaculorization. Additional phenotypes such as proteolysis of cytokeratins, increased caspase activity, increased expression of TM1, loss of adherens junctions, and an increase in apoptosis along the villus axis suggest that the intestinal epithelium of the Myo1a/villin KO mouse is undergoing anoikis. This was supported by the American Cancer Society, NIH, and Crohn's and Colitis Foundation.

317/B261

A Novel Role for a Class 1 Myosin during Mitosis.

A. Rump[1], T. Scholz[2], G. Tsiavaliaris[1]; [1]Biophysical Chemistry, Hannover Medical School, Hannover, Germany, [2]Molecular and Cell Physiology, Hannover Medical School, Hannover, Germany

Myosin-1C is one of three subclass-Iα myosins produced in Dictyostelium discoideum. Subclass-Iα myosins have a long tail composed of three distinct regions referred to as tail homology (TH) domains 1, 2 and 3. We investigated the cellular function of myosin-1C and identified a novel role for this myosin during mitosis. In interphase myosin-1C is involved in the formation of endocytic cups and membrane protrusions. At the onset of mitosis, the protein relocalizes resulting in a predominant centrosomal localization during prophase. The centrosomal association persists during later stages of mitosis when additionally a strong localization of myosin-1C along the mitotic spindle microtubules (MTs) is observed. After mitotic spindle breakdown in late telophase, myosin-1C again associates with actin-rich structures in the cell periphery. In vitro TIRF microscopy experiments with different myosin-1C tail domain constructs and MT-cosedimentation assays demonstrate a direct association of myosin-1C with MTs. Only constructs containing both TH1 and TH2 bind efficiently to MTs under the assay conditions. Additionally, our results show that the myosin-1C tail domain promotes MT assembly, prevents MT-depolymerisation, and crosslinks MTs to F- actin. A stabilizing effect of myosin-1C is also suggested from the results of studies with myosin-1C knockout cells. These cells exhibit reduced MT densities at the mitotic spindle, a reduced astral MT size and number, and a reduced proliferation rate in comparison to wild-type cells. In addition we observe that myosin-1C constructs lacking the motor domain display a permanent centrosomal localization, whereas the motor domain alone evenly distributes in the cytosol during the entire cell cycle. Our results suggest that myosin-1C motor activity is required for the relocalization of the protein, while the tail domain of myosin-1C interacts with MTs and F-actin and is required to bind and position the mitotic spindle.

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Regulation of Myosin IIIA Motor Activity by Intermolecular Autophosphorylation.

J. E. Moore[1], A. C. Dosé[2], B. Burnside[2], C. Yengo[1]; [1]Biology, UNC-Charlotte, Charlotte, NC, [2]Molecular and Cell Biology, UC-Berkeley, Berkeley, CA

Myosin IIIA is a motor protein associated with the actin bundles of photoreceptors and inner ear hair cells. Interestingly, myosin IIIA contains an N-terminal serine-threonine kinase domain capable of autophosphorylating sites on its motor. To examine autophosphorylation as a potential means of regulating motor function we have developed a kinase-dead construct, MIIIA K50R. We have compared its ATPase and actin-binding properties to those of the wild-type MIIIA construct in the phosphorylated and unphosphorylated states. We have also compared the cellular localization of GFP-tagged kinase-dead myosin IIIA to that of the wild type in transfected COS cells. Further, we investigated autophosphorylation rates under a range of myosin concentrations and in the presence versus the absence of actin. We have determined that inactivation of the myosin IIIA kinase reduces the K_{ATPase} (actin concentration at half V_{max}, k_{cat}) (MIIIA ~34 μM; MIIIA K50R ~11.9 μM) and enhances steady-state affinity for actin (MIIIA ~7.0 μM; MIIIA K50R ~5.3 μM) as compared to wild type, yet it does not affect k_{cat} (MIIIA ~0.77 sec^{-1}; MIIIA K50R ~0.76 sec^{-1}). Full phosphorylation of MIIIA results in a ~40% reduction in ATPase activity compared to unphosphorylated MIIIA, in the presence of 20 μM actin. Transient kinetic experiments suggest that the equilibrium constant between actomyosin ADP states (K'_{5A}) may be the key step altered in the MIIIA K50R cycle. Phosphorylation rates are unchanged by the presence of actin but have been found to be dependent upon myosin IIIA concentration within the range of 0.1-1.2 μM (~0.05 min^{-1} - ~0.29 min^{-1}), indicating that autophosphorylation occurs intermolecularly. The kinase-dead MIIIA construct localizes more efficiently to the tips of filopodia in COS cells than does wild type, although not as strikingly as a previously studied kinase-removed MIIIA construct. Our results suggest intermolecular autophosphorylation regulates myosin IIIA motor activity and its degree of localization to the tips of actin bundles in sensory cells.

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Kinase Activity and Autophosphorylation of Mouse Class III Myosins.

J. Dalal[2], K. E. Kempler[1], N. Munoz[1], S. Alvarez[3,4], S. McClung[3], A. Dose[2], B. Burnside[2], B. Battelle[1]; [1]Whitney Laboratory for Marine Bioscience, University of Florida, St. Augustine, FL, FL, [2]Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, [3]Proteomics Core, ICBR, University of Florida, Gainesville, FL, [4]Donald Danforth Plant Science Center, St. Louis, MO

With a kinase domain at their N-terminus, class III myosins probably exhibit both signaling and motor functions, and class III myosins may be modulated by autophosphorylation. To better understand the role of class III myosin autophosphorylation, we are examining the substrate specificities of the kinase domains of mouse myosin 3A and 3B (Myo3A and Myo3B) and identifying autophosphorylation sites. **Methods:** Kinase domains of mouse Myo3A and Myo3B were expressed in sf9 cells. Kinase activity was assayed by quantifying the incorporation of 32P-ATP into substrates. Phosphorylated amino acids were identified using standard protocols and phosphorylated sites were identified by tandem mass spectrometry. **Results:** Both kinases autophosphorylate their kinase domains and exhibit similar relative abilities to phosphorylate a variety of exogenous substrates. Predicted PKA and PKC sites are phosphorylated by both kinases. Myo3A and Myo3B kinases also phosphorylate the motor and tail domains of Myo3B. Two conserved phosphorylation sites are located within the kinase domains of Myo3A and Myo3B: the activation threonine that typically autophosphorylates in kinases and a predicted PKC site on the N-terminal side of the activation threonine. Threonine autophosphorylation is greatly preferred within the kinase domain; it is not yet clear whether the serine is also autophosphorylated or phosphorylated by a different kinase. Autophosphorylation sites within the motor domain have not yet been identified; however, Myo3A and Myo3B kinases phosphorylate serines and threonines in a peptide within the tail homology domain of Myo3B. **Conclusions:** The kinase domains of Myo3A and Myo3B have broad and similar substrate specificities. The kinase, motor and tail domains of Myo3B can be autophosphorylated, and Myo3A kinase can phosphorylate the myosin and tail domains of Myo3B. Thus, each domain of class III myosins may be modulated by autophosphorylation, and if Myo3A and Myo3B colocalize in cells, each may phosphorylate the other.

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Myosin-Va in RNA Distribution: No Motor Domain Required.

V. P. Salerno[1,2], D. Provance[1], C. M. Silan[1], J. R. Sotelo-Silveira[3], J. R. Sotelo[3], J. Mercer[1]; [1]McLaughlin Research Institute, Great Falls, MT, [2]Departamento de Biociências da Atividade Física - EEFD, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil, [3]Departamento de Proteínas y Ácidos Nucleicos, Instituto de Investigaciones Biologicas Clemente Estable, Montevideo, Uruguay

Myosin-Va has been implicated in RNA transport and/or distribution in neurons and fibroblasts. In this study, we show that a small, 9-base exon in the cargo-binding tail domain of myosin-Va, also required for binding to dynein light chain 2 (DLC2 or LC8), is required for rescue of the null mutant RNA distribution phenotype in fibroblasts. DLC2/LC8 colocalizes with a subset of newly-synthesized RNA in both the nucleus and cytoplasm, and overexpression of the myosin-Va tail reduces DLC2/LC8 levels in the nucleus. We also show that the motor, or head, domain is not required for rescue. These data suggest that myosin-Va interacts with RNA, but does not actively transport it. Instead, it appears to have a more passive role as a passenger on particles or organelles that are moved along microtubules.

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An Ex Vivo Motility System Reveals Regional Control of Unconventional Myosin Motor Activity.

C. M. Brawley, R. S. Rock; Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL

Eukaryotic cells have a self-organizing cytoskeleton, where motors transport cargoes on these cytoskeletal tracks. Since we have already demonstrated that myosin X can select for motility based on actin architecture (Nagy et al., PNAS:105(28), 2008), we suggest that any myosin motor may possess this ability of selection. To understand the sorting processes, we describe a system to observe single-molecule traffic patterns of multiple motor types in a cellular context. We followed the motility of class V, VI, and X myosins on preserved actin cytoskeletons from Drosophila S2 cells. From the trajectory maps, we find a largely radial actin architecture, but with a subset of filaments having the opposite polarity (barbed ends toward the cell center). We also see regional control of motility for all three motors, with several preferred regions (with varying velocity) and dead-zones. Any of these features are not apparent from conventional in vitro motility assays using purified components. We further compared our S2 trajectory maps to trajectory maps of myosin V, VI and X created in Cos-7 and U2OS cells. These mammalian cell types display a more complex actin architecture, revealed dramatically by the trajectory maps. Cos-7 cells have more isotropic actin than S2 cells and support motility of all three classes of myosins. U2OS cells hardly support any myosin motor motility. Although, myosin VI does make significantly more processive runs than myosin V and X, and these myosin VI motor runs are specific for the stress fiber cords that span the length of the U2OS cells. Interestingly, the velocities for all three classes of myosins are similar for S2 and the mammalian cell types analyzed. This conclusion supports that myosin motility is selected for based on the actin architecture of cells.

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Myosin Vc Is a Molecular Motor That Functions in Secretory Granule Trafficking.

D. Jacobs[1], R. Weigert[2], J. G. Donaldson[3], R. Cheney[1]; [1]Cell and Molecular Physiology, University of North Carolina-Chapel Hill, Chapel Hill, NC, [2]National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, [3]Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD

Class V myosins are actin-based motor proteins that have critical functions in organelle trafficking. Of the three class V myosins expressed in mammals, relatively little is known about the cell biological functions of Myo5c. Here we use MCF-7 cells to visualize the dynamics of Myo5c in living cells, identify the organelles that native Myo5c associates with, and test the functions of Myo5c. Immunofluorescence demonstrates that endogenous Myo5c localizes to small puncta and to slender tubules. In migrating cells, Myo5c puncta exhibit a highly polarized distribution near the leading edge. Live-cell imaging shows that puncta of GFP-Myo5c move slowly (~30 nm/s), are insensitive to nocodazole, and can be imaged for extended periods (up to 38+ minutes). The tubules move rapidly (~500 nm/s), are sensitive to nocodazole, and colocalize with antibodies to exo70. Importantly, Myo5c puncta colocalize with markers for organelles that undergo regulated secretion. The Myo5c forms a ring around the granule markers, which is consistent with it localizing to the cytoplasmic surface of the secretory granule. Two-color TIRF + DIC imaging shows that these granules can be triggered to undergo secretion. Interestingly, although exocytosis of granule contents occurred in 3-6 seconds, the Myo5c fluorescence remained detectable for an additional 10-60 s. Finally, expressing a dominant negative Myo5c tail construct perturbs the distribution of secretory granule markers. These results provide the first high resolution studies of Myo5c localization and dynamics in a cell line and indicate that Myo5c functions in the trafficking of secretory granules. (This research was supported by a Porter Fellowship from the APS and a UNC Sequoyah Dissertation Fellowship to DTJ and a NIH-NIDCD grant DC03299 to REC).

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The Conditional Processive Mechanism of Dictyostelium Myosin-5b Is Required for Contractile Vacuole Function.

M. H. Taft, F. K. Hartmann, A. Rump, I. Chizhov, D. J. Manstein, G. Tsiavaliaris; Institute for Biophysical Chemistry, Medical School Hannover, Hannover, Germany

Several members of the myosin-5 family are characterized by a high duty ratio, which enables them to move as single molecules over long distances along actin filaments without dissociating. Here, we investigated the functional role and kinetic properties of myosin-5b from Dictyostelium discoideum and show that this myosin can switch between processive and non-processive movement under physiological conditions. In Dictyostelium cells myosin-5b is enriched at highly dynamic vesico-tubular structures in the cell periphery corresponding to the contractile vacuole (CV) system. The cellular localization of the CV system and the tight association of myosin-5b with the CV system facilitate the direct observation of changes in myosin-5b motor activity and their correlation with changes in the concentration of free Mg2+-ions by TIRF microscopy. To investigate the functional importance of myosin-5b at the CV system, we have exposed Dictyostelium cells expressing wild-type and a dominant negative mutant variant of myosin-5b to high and low osmotic pressure and show that myosin-5b is directly involved in the active contraction of the CV system. Our results from kinetic analyses and in vitro motility assays demonstrate that the processive properties of myosin-5b depend on physiological changes in the concentration of free Mg2+-ions. Free Mg2+-ions strongly

affect the release of hydrolysis products thereby influencing the fraction of time the motor spends in the strong actin binding states. To dissect the physiological relevance of the observed Mg2+-dependent functional changes of myosin-5b, we followed the spatial and temporal changes in free Mg2+-ion distribution in Dictyostelium cells. Using the Mg2+-sensitive fluorescent dye KMG-104AM, we observed fluctuations in the concentration of free Mg2+-ions that take place in the millisecond to second range and thus within a time domain where the consequences for myosin motor activity are of physiological relevance.

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Alternative Splicing in Class V Myosins Determines Association with Rab10.

J. Roland, L. A. Lapierre, J. Goldenring; Surgery, Vanderbilt University Medical Center, Nashville, TN

Rab proteins influence vesicle trafficking pathways through the assembly of regulatory protein complexes. Previous investigations have documented that Rab11a and Rab8a can interact with the tail region of Myosin Vb and regulate distinct trafficking pathways. We have now determined that a related Rab protein, Rab10, can interact with Myosin Va, Myosin Vb, and Myosin Vc. Rab10 localized to a system of tubules and vesicles that have partially overlapping localization with Rab8a. Both Rab8a and Rab10 were mislocalized by the expression of dominant-negative Myosin V tails. Interaction with Rab10 was dependent on the presence of the alternatively spliced exon D in Myosin Va and Myosin Vb and the homologous region in Myosin Vc. Yeast two-hybrid assays and FRET studies confirmed that Rab10 binding to Myosin V tails in vivo required the alternatively spliced exon D. In contrast to previous work, we found that Rab11a can interact with both Myosin Va and Myosin Vb tails independent of their splice isoform. These results indicate that Rab GTPases regulate diverse endocytic trafficking pathways through recruitment of multiple Myosin V isoforms.

325/B269

Cargo Switching at Actin-Microtubule Intersections: A Case for Strength in Numbers.

H. W. Schroeder, H. Shuman, E. L. Holzbaur, Y. E. Goldman; Pennsylvania Muscle Institute, University of Pennsylvania School of Medicine, Philadelphia, PA

Active switching of cargo between actin filaments (AFs) and microtubules (MTs) is a key aspect of endocytosis and secretion. Both the MT-dependent motors, kinesins and dyneins, and the AF-dependent motors, myosins, are often found bound to the same organelle. Recent studies suggest that there may be an active tug-of-war between AF motors and MT motors for their respective cytoskeletal tracks. Regulation of the activity and/or cargo affinity of the motors may determine whether the cargo passes, pauses, or switches at AF-MT intersections. We have tested the tug-of-war hypothesis in vitro by utilizing an optical trap to position bead cargos with 1-4 actively engaged motors near actin-microtubule intersections; this procedure has allowed us to examine cytoskeletal switching as a function of motor number. We show that the number of motors can be used to regulate switching behavior. When the stall force measured with an optical trap for myosin V and dynein-dynactin is equivalent, the probability of the cargo switching or remaining on its respective track is about equal; under these conditions, approximately 30% of these cargos become tethered at the intersection. At force ratios greater than two, the bead cargo rarely becomes tethered at the intersection and instead switches with ~70% probability to the filament whose motor group is exerting greater force. Interestingly, rotation of the cargo is often seen specifically at the intersections which implies a torque component and suggests a mechanism for switching. Supported by P01 AR051174.

326/B270

Step Size and Processivity of Myosin VI Dimers with Engineered Artificial Lever Arms.

M. W. Elting[1,4], Z. D. Bryant[2], J. Liao[3], J. Spudich[1]; [1]Biochemistry, Stanford University, Stanford, CA, [2]Bioengineering, Stanford University, Stanford, CA, [3]Mechanical Engineering, Columbia University, New York, NY, [4]Applied Physics, Stanford University, Stanford, CA

Dimeric myosin VI moves processively toward the (-) end of actin filaments with a large and highly variable step size. The lever arm of myosin VI has an unusual structure, containing only two light chains and a single alpha helical domain. The structural and mechanical properties of the lever arm are thought to be important for mediating intrahead communication, ensuring processive motion, and determining the distribution of step sizes. We have engineered highly processive dimers in which large portions of the lever arm are replaced by alpha-actinin repeats, demonstrating that finely tuned mechanical properties are not critical for processive motion. We are examining the effects of this artificial lever arm on the step size and processive run length of myosin VI, in order to rigorously challenge quantitative models of processive myosin stepping.

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Structural and Mechanistic Determinants of Myosin VI Processivity.

P. Chuan[1], A. R. Dunn[1], Z. D. Bryant[2], J. Spudich[1]; [1]Biochemistry, Stanford University School of Medicine, Stanford, CA, [2]Bioengineering, Stanford University School of Medicine, Stanford, CA

Myosin VI is a motor that takes many consecutive 36 nm steps along an actin filament. The parameters necessary for such processive movement are not fully understood. We are using high-speed gold nanoparticle tracking to study single molecules of myosin VI with millisecond resolution in the absence of load. Our measurements of rapid detached head rebinding rates and processive run lengths for wild-type and mutant tail domain constructs support a simple model of processive stepping, and challenge the view that robust communication between the two heads is essential for processivity.

328/B272

Insights into Cortical F-actin Polarity and Endocytic Trafficking Using In Vitro Assays with Myosin VI Coated Nanospheres on Detergent Extracted Keratocytes.

S. Sivaramakrishnan, A. Dunn, J. Spudich; Biochemistry, Stanford University, Stanford, CA

Myosin VI, an actin based molecular motor, has been shown to localize to endocytic vesicles and plays an important role in their internalization through the F-actin cortex. This involves the interaction of multiple myosin VI motors on endocytic vesicles with the F-actin meshwork in the cell periphery. Our current model of filament polarity in the cortical F-actin cytoskeleton is based on studies that are limited to the tips of the lamellipodia in spreading cells. Fish epidermal keratocytes have a dense and extensive polarized F-actin meshwork. We model the myosin VI mediated endocytic process using an in vitro assay involving myosin VI coated nanospheres moving on detergent extracted keratocytes. Although a single myosin VI molecule is a weakly processive motor (run length ~ 300 nm), we find that multiple myosin VI motors enhance processivity greater than 10-fold. Myosin VI isolated from cells is a monomer but has been reported to dimerize in vivo. We find that multiple myosin VI monomers or artificial dimers are capable of moving myosin VI coated nanospheres along single actin filaments in vitro. However, ten-fold fewer dimers than monomers are required to move nanospheres through the same distance and they do so with five-fold higher velocity. Nanospheres coated with myosin VI dimers move effectively along extracted keratocytes with large run lengths (> 5 μm) whereas myosin VI monomers are unable to navigate the extracted keratocyte cytoskeleton. Despite the highly branched F-actin structure in keratocytes, myosin VI dimer coated nanospheres preferentially move along relatively straight trajectories from the cell edge to the center. A computational model of myosin VI movement on keratocyte F-actin shows that such movement is possible when F-actin filaments throughout the lamellipodia and lamella are polarized with their minus ends pointed towards the cell center. Additionally, our model predicts that myosin VI dimers need to switch with probability of 50% at filament junctions in order to enable continuous movement towards the center. Our studies provide a detailed polarity road map of the cortical F-actin cytoskeleton with insights into how myosin VI moves endocytic vesicles in vivo.

329/B273

A Stable Single α-helix Can Act as a Flexible Lever in Myosins.

T. Baboolal[1], T. Sakamoto[2], G. Mashanov[3], J. Sellers[2], J. Molloy[3], P. J. Knight[1], M. Peckham[1]; [1]University of Leeds, Leeds, United Kingdom, [2]NHLBI, NIH, Bethesda, MD, [3]NIMR, London, United Kingdom

Myosins convert energy from ATP hydrolysis into mechanical work using a neck region to amplify the small conformational change in their motor domain. We recently showed that the predicted coiled-coil domains of myosin 10, myoM, myosin 6 and myosin 7a are likely to form a stable single α-helix (SAH) that could lengthen the lever resulting in larger steps. Using a bio-informatics approach, we also identified SAH domains in many other proteins other than myosins. We have also shown that the predicted coiled-coil domain of myosin 10 is actually a SAH domain, and that this myosin is monomeric, not dimeric as originally predicted (Knight et al., 2005. J.Biol. Chem.) To test if the predicted coiled-coil domain of MyoM is also a SAH domain, we expressed and purified a myosin 5 HMM SAH domain chimaera in which 4 of the 6 calmodulin binding motifs were replaced by the SAH domain of MyoM (112 aa), such that the length of the lever was maintained (2IQ-SAH-HMM). In a single molecule total internal reflection fluorescence microscopy (TIRFM) assay, the GFP-labelled 2IQ-SAH-HMM construct moved processively at similar velocities to 6IQ-HMM, with similar step size but a broader distribution (66.5 + 15.8nm compared to 75.3 + 9.3nm). Electron microscopy of rotary and unidirectionally shadowed preparations showed monomers with a clearly visible SAH domain between the head-tail junction and the heads of myosin 5, a consistent with an α-helix of 112 amino acids. Together with our previous work (Knight et al., JBC 2005) and that of others (Spink et al., 2008, NSMB) on myosin 6, we now know that the predicted coiled-coil domains of myosins 6 and 10, and MyoM are SAH domains. We have recently followed up this work by performing experiments in live cells, in which we visualised the movement of GFP-tagged full length myosin 10 in filopodia by TIRFM. Preliminary experiments with mutant myosin 10 in which the SAH domain has been deleted, suggests that this deletion alters its velocity. Therefore, the SAH domain appears to contribute to lever function in myosins.

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An Unconventional Myosin Is Essential for Chemotaxis.

L. Breshears[1], D. Wessels[2], D. Soll[2], M. Titus[1]; [1]University of Minnesota, Minneapolis, MN, [2]University of Iowa, Iowa City, IA

The social amoeba *Dictyostelium discoideum* is a powerful model system for dissecting chemoattractant signaling pathways and identifying the cytoskeletal components necessary for directed cell migration. MyoG is a novel unconventional myosin characterized by two MyTH/FERM domains in its tail region. The potential role of this myosin in *Dictyostelium* motility was investigated by analyzing the phenotype of two independent *myoG* null mutants in detail. The initial stages of development, induced by starvation, depend on chemotaxis to cAMP, resulting in the formation of a multi-cellular aggregate. Upon starvation *myoG* null mutants fail to aggregate, arresting as a smooth monolayer of cells. Starvation induces the expression of genes necessary for chemotaxis and aggregation. The *myoG* null mutants express these genes normally, indicating that they are able to respond correctly to starvation. MyoG is not required for trafficking of the chemotaxis receptor (cAR1) as the GFP-cAR1 receptor is localized to the cell periphery in the null mutants. These data suggest that the defect in the mutants lies in the chemotactic response downstream of the receptor, so a quantitative analysis of *myoG* null mutant motility and chemotaxis was performed. The *myoG* null mutants neither polarize in a cAMP gradient nor do they increase cell speed, as observed for wild type cells. Also, *myoG* null cells do not chemotax toward the cAMP source but do exhibit a slight increase in persistent, though directionally random, movement. Analysis of the ability of *myoG* null cells to polymerize actin in response to cAMP revealed that the response is dampened in the mutants. These data show that while the mutant cells retain some ability to respond to the gradient, the major pathways regulating polarity and chemotaxis are not functional. The mutant phenotype suggests that MyoG acts in transducing the chemotactic signal from the cAMP receptor to the actin cytoskeleton, facilitating the morphological changes that lead to polarization and directional migration.

331/B275

A Role for Myosin IXb, a Motor-Rho-GAP Chimera, in Epithelial Wound Healing.

S. K. Chandhoke, M. Mooseker; Molecular Cellular and Developmental Biology, Yale University, New Haven, CT

Myosin IXb (Myo9b) has been implicated in inflammatory bowel diseases (IBD) including Crohn's disease, celiac disease and ulcerative colitis. This single-headed, processive motor contains a Rho GTPase activating protein (Rho-GAP) domain within its tail region, suggesting its involvement in Rho-mediated actin cytoskeletal modifications critical to intestinal epithelial barrier function and regulation. Although the

motor properties of Myo9b have been studied, less is known regarding its cellular functions. Epithelial wound healing is a dynamic and coordinated process of cell migration that is essential to tissue repair and maintenance in both pathological and healthy states. In differentiated monolayers of the intestinal epithelial cell line Caco2BBe (BBe), we have observed a striking redistribution of Myo9b upon wound healing. Wounded regions of BBe cell monolayers de-differentiate, with Myo9b specifically localizing to extreme leading edges of migrating cells, co-localizing with filamentous actin. BBe cells expressing the C-terminal tail tip domain of Myo9b fail to migrate into the wound, a result which provides the first evidence that the tail tip may play a key role in Myo9b localization and/or regulation of its GAP domain. Interestingly, formation of lamellar extensions in non-expressing cells adjacent to tail tip-expressing cells is inhibited, suggesting that the intercellular coordination required for wound healing is impaired. In addition, RNAi knockdown of Myo9b expression in HeLa cell monolayers results in a marked inhibition of scratch-wound induced rearrangements of the actin cytoskeleton at the wound margin. This includes loss of lamellipodial extensions and maintenance of focal adhesions. Instead, Myo9b knockdown cells at the wound margin form stress-fiber like arrays of actin at the free edges of cells facing the wound. These data reveal a critical and cellular role for Myo9b during epithelial wound healing, which may project to its implication in disorders of the gastrointestinal tract. This work is supported by the NIH and the Crohn's and Colitis Foundation of America.

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Myosin-X Is Required for Proper Behavior of Neural Crest Cells in Xenopus laevis.

Y. Hwang, T. Luo, Y. Xu, T. D. Sargent; LMG, NIH/NICHD, Bethesda, MD

Xenopus unconventional, non-muscle myosin, MyoX has been sorted out in a screen for genes expressed in neural crest (NC) under the control of the transcription factor TFAP2. At neurula stage MyoX has the highest level of expression in the NC, but is also abundant in paraxial mesoderm and all the brain primordia. A couple of splice-inhibitory MyoX morpholinos were designed by mapping intron locations onto the Xenopus laevis sequence based on the structure of the Xenopus tropicalis MyoX gene, and were confirmed in partial knock down. MyoX knockdown in vivo using these splice-inhibitory morpholinos resulted in identical phenotypes showing retarded migration of cranial NC cells into arches, subsequent reduction of head and jaw cartilage and reduction in the NC-derived trigeminal nerve. NC cells migrate most efficiently on fibronectin, utilizing integrin alpha5beta1 as the receptor. In vitro migration assays on fibronectin using explanted cranial neural crest cells showed significant inhibition of cell attachment, spreading and migration resulting from MyoX knockdown. Rhodamin-phaloidin staining visualized malformation of membrane ruffle, lamellipodia and filopodia among these cells. It has been reported that the FERM-domain of MyoX physically interacts with a conserved NPXY motif present in the cytoplasmic domain of most beta integrins. In live-cell imaging, the transient colocalization of Integrin beta1 and MyoX at filopodial tips was observed in migrating CNC cells expressing GFP-MyoX / Integrin-mCherry, but not GFP-headless MyoX / Integrin-mCherry. These aberrant cytoskeletal features were not reversible even by forced activation of Integrin with divalent Manganese ion suggesting that MyoX functions in NC cells in regulating beta1-integrin distribution.

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Single Molecule Investigation of the Acto-Myosin-10 Complex Using Optical Tweezers.

Y. Takagi[1], R. E. Farrow[2], G. I. Mashanov[2], C. Batters[3], Y. Yang[1], M. Peckham[4], J. R. Sellers[1], J. E. Molloy[2]; [1]LMP, NHLBI, National Institutes of Health, Bethesda, MD, [2]Division of Physical Biochemistry, MRC National Institute for Medical Research, London, United Kingdom, [3]Laboratory of Molecular Biology, The Medical Research Council, Cambridge, United Kingdom, [4]Institute of Molecular and Cellular Biology, University of Leeds, Leeds, United Kingdom

Recent cell biological studies of myosin-10 have revealed that myosin-10 is essential to cellular processes such as filopodia extensions and phagocytosis. Steady-state and transient kinetic biochemical studies of the ATPase cycle of a single-headed, subfragment-1 like (S1), construct of myosin-10 show that it has an intermediate duty-cycle ratio. It remains tightly bound to actin for about 16% of its total ATPase cycle time but spends around 90% associated with actin in both weak and strongly bound states. Furthermore, the acto-myosin-10-S1 complex has two ADP bound states, as well as a surprisingly low affinity for actin, comparable to that of the rigor complex between actin and skeletal muscle myosin II. In order to study the mechano-chemical coupling of myosin-10, we used the actin filament sliding assay and a dual-beam, optical tweezers apparatus to perform single molecule mechanical studies. For these studies we used a two-headed, heavy meromyosin-like (HMM) construct of myosin-10 that contained a leucine-zipper at the C-terminal end to force dimerization of the protein. In this assay, myosin-10-HMM moves filaments at a velocity of ~125 nm s^{-1}. This is similar to the speed of intact, GFP-tagged, myosin-10 moving within filopodia of live mammalian cells at room temperature (~140 nm s^{-1}) measured by TIRF microscopy and single-particle tracking. Optical trapping results showed that the average powerstroke size was ~10 nm, with the rate of ATP binding of ~1.3 μM^{-1} s^{-1}. In most of the raw data traces, we observed displacements of unitary size, however at low ATP concentrations (2 μM) we also observed a number of interactions that exhibited multiple, staircase-like movements consisting of up to 3 steps per binding interaction. This behaviour is characteristic of a *processive* molecular motor. In order to probe the stiffness of the acto-myosin-10-HMM complex we applied a small force by moving the optical tweezer laterally and measured the resulting displacement. We will discuss these measurements in the context of mechano-chemical coupling and the functional significance in the living cell.

334/B278

Myosin-10 and Actin Filaments Are Essential for Mitotic Spindle Function.

S. Woolner[1,2], L. O'Brien[3], C. Wiese[3], W. Bement[1]; [1]Department of Zoology, University of Wisconsin-Madison, Madison, WI, [2]Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom, [3]Department of Biochemistry, University of Wisconsin-Madison, Madison, WI

The role of actomyosin in the mitotic spindle has long been a subject of controversy. Here we present evidence that Myosin-10 (Myo10), an actin-based motor that binds microtubules, participates with actin filaments (F-actin) to regulate mitotic spindle function. Using Xenopus laevis early embryos as an in vivo system to study mitosis and Xenopus egg extracts to assemble mitotic spindles in vitro, we show that Myo10 localizes to the mitotic spindle where it concentrates at the spindle poles from prometaphase through to the end of telophase.

Knockdown of Myo10 levels in vivo using an anti-sense morpholino approach reveals that Myo10 is essential for 4 aspects of mitotic spindle function: proper spindle anchoring, spindle length maintenance, spindle pole integrity, and progression through metaphase. Furthermore, by using live imaging we show for the first time that highly dynamic F-actin cables are present in the mitotic spindle and that these cables both surround the spindle and extend between the spindle and the cell cortex. Remarkably, we find that although proper spindle anchoring depends on both F-actin and Myo10, the requirement for Myo10 in spindle pole integrity is F-actin independent, while F-actin and Myo10 actually play antagonistic roles in maintenance of spindle length. Our results show that Myo10 and F-actin play critical roles in mitotic spindles and that Myo10 and F-actin have unexpected interdependencies.

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An Actin-binding Site Is Located in the Tail Domain of Myo1, a Class XIV Myosin in Tetrahymena thermophila.

M. Gotesman[2], R. E. Hosein[1], R. H. Gavin[1,2]; [1]Biology, Brooklyn College - CUNY, Brooklyn, NY, [2]Biology Doctoral Program, CUNY Graduate School, Brooklyn, NY

Myo1 is one of 12 myosins initially assigned to myosin class XX but recently re-assigned to a subclass within class XIV. The Myo1 tail contains a FERM domain (band 4.1-ezrin-radixin-moesin homology domain). FERM domains are present in several cytoskeletal proteins and are known to interact with the actin cytoskeleton. Previous studies have shown that Myo1 and actin localize to phagosomes and are involved in phagosome motility. We reasoned that an actin-binding site in the FERM domain of the Myo1 tail facilitates interaction of Myo1 with phagosomes and the actin cytoskeleton. In order to search for an actin-binding site in the Myo1 tail, a tagged truncated MYO1 tail domain was created by adding a GFP (Green Fluorescence Protein) sequence to the 5' end of the last 1362 nucleotides (including the 924-nucleotide FERM domain) of the MYO1 tail. The truncated MYO1 sequence was cloned into an expression vector containing a metallothionein promoter, and the vector was used to transform Tetrahymena by biolistic bombardment. An immunoblot of total cell lysate from GFP-expressing cells was probed with anti-GFP antibody, which confirmed expression and stability of the 80-kDa GFP-Myo1 truncated tail. Anti-actin antibody pulled down actin and its binding partners from total lysate of GFP-expressing cells. Immunoblots of pull-down fractions detected GFP-Myo1 truncated tail, actin, and endogenous Myo1 when probed respectively with anti-GFP, anti-actin, and anti-Myo1 antibodies. The GFP-Myo1 truncated tail localized to phagosomes, nucleus, and basal bodies consistent with previously reported localization of full-length Myo1. We conclude that localization of Myo1 is achieved through its tail domain, which contains an actin-binding site for putative interactions with actin-rich regions of the cytoskeleton. [Supported by National Science Foundation]

Actin-Associated Proteins I (336-360)

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Distinct Roles for Arp2/3 Regulators in Actin Assembly and Endocytosis.

B. Galletta, D. Chuang, J. Cooper; Cell Biology and Physiology, Washington University School of Medicine, Saint Louis, MO

The Arp2/3 complex is a critical component required for the formation of dendritic actin networks. Arp2/3 can generate branches and seed new filaments. The proper regulation of Arp2/3 activity is likely to be critical for efficient generation of force and movement by actin networks, suggested in part by the presence of several potential Arp2/3 regulators in many cells. Outstanding questions in our understanding of the dendritic nucleation model are how the regulation of Arp2/3 activity, by a variety of regulatory proteins, specifically influences actin network formation and function, and how the activities of these regulators are coordinated or overlap in vivo? We have utilized the yeast actin patch as a model system for actin assembly and movement. Actin patches, which correspond to sites of endocytosis, contain a branched, Arp2/3 dependant actin network and undergo a series of stereotypical changes in protein composition and motility. Mutations affecting all the yeast Arp2/3 regulators, Las17/WASp, the type-I myosins, Pan1, Abp1, and Coronin (Crn1), were studied. The assembly and movement of GFP-labeled patch proteins, whose movements define the major phases of patch motility, were followed using high-speed confocal microscopy, coupled with computer-assisted particle tracking and quantitative motion analysis. This approach has allowed us to determine the effects of improper Arp2/3 regulation on actin network assembly and patch movement and to detect several previously unappreciated phenotypes. We find that Arp2/3 regulators have both distinct and overlapping roles in patch formation, the initiation of movement away from the membrane, and the movement of the patch into the cytoplasm. To better understand the molecular mechanisms by which these Arp2/3 regulators are influencing network formation, we have undertaken studies to determine how mutation of the Arp2/3 regulators effects the composition of dendritic nucleation proteins within the patch, over time, using quantitative, live cell, fluorescent microscopy.

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The WAVE/SCAR Complex Promotes Subcellular F-actin Enrichment and Maintains Apical/basal Polarity during C. elegans Embryogenesis.

M. Soto, F. Patel, Y. Bernadskaya; Pathology, Robert Wood Johnson Medical School, Piscataway, NJ

Establishment of apical/basal polarity requires organization of the actin cytoskeleton. However, the cell movements of morphogenesis require re-organization of actin. Our objective is to identify the mechanisms by which cytoskeletal and apical/basal organization are maintained during dynamic movements. The WAVE/SCAR complex promotes actin nucleation through the Arp2/3 complex, in response to Rac signaling. We have shown that loss of WVE-1/GEX-1, the only C. elegans WAVE/SCAR homolog, by genetic mutation or by RNAi, has the same phenotype as loss of GEX-2/ Sra1/p140/PIR121, GEX-3/ NAP1/HEM2/KETTE, or ABI-1/ABI, the three other components of the C. elegans WAVE/SCAR complex. We find that the entire WAVE/SCAR complex promotes actin-dependent events at different times and in different tissues during development. During *C. elegans* embryogenesis loss of CED-10/Rac1, WAVE/SCAR complex components, or Arp2/3, blocks epidermal cell migrations despite correct epidermal cell differentiation. 4D movies show that this failure occurs due to decreased membrane dynamics in specific epidermal cells. Unlike myoblasts in *Drosophila*, epidermal cell fusions in *C. elegans* can occur in the absence of WAVE/SCAR or Arp2/3. Instead we find that subcellular enrichment of F-actin in epithelial tissues requires the Rac-WAVE/SCAR- Arp2/3 pathway. Intriguingly, we find that at the same stage of development both F-actin and WAVE/SCAR proteins are

enriched apically in one epithelial tissue and basolaterally in another. Thus temporally and spatially regulated actin nucleation by the Rac-WAVE/SCAR- Arp2/3 pathway is required for epithelial cell organization and movements during morphogenesis. We further find that epithelial cells in developing embryos can establish apical/basal polarity in the absence of this actin nucleation pathway, but polarity is progressively compromised. This suggests a continuous and dynamic requirement for Arp2/3-dependent actin nucleation to maintain polarity during development. *Supported by grants from the American Heart Association and the National Science Foundation.*

338/B282

IQGAP3 Regulates Cell Proliferation through the Ras/ERK Signalling Cascade.

H. Nojima[1], M. Adachi[2], S. Tsukita[2], S. Tsukita[1]; [1]Biological Science, Osaka University, Suita, Japan, [2]Department of Cell Biology, Kyoto University, Kyoto, Japan

Proliferation of epithelial cells must be spatiotemporally regulated to maintain the organization of epithelial sheets. Here we show that the IQGAP family, comprising IQGAP1, 2 and 3, underlies lateral cell-cell contacts of epithelial cells. Of the three proteins, IQGAP3 is unique in that its expression is specifically confined to proliferating cells. Knockdown of IQGAP3 in cultured epithelial cells caused inhibition of proliferation and ERK activity. When exogenously expressed in quiescent cells, IQGAP3 was capable of inducing cell-cycle re-entry, which was completely inhibited by the MEK inhibitor U0126. Thus, IQGAP3 is necessary and sufficient for driving cell proliferation and ERK acts downstream of IQGAP3. Furthermore, IQGAP3 specifically interacted with the active, GTP-bound form of Ras, and in IQGAP3 knockdown cells, the activity of Ras, but not of other small GTPases, was inhibited. Thus, IQGAP3 regulates the promotion of cell proliferation through Ras-dependent ERK activation.

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Regulation of the Actin Cytoskeleton by dip1p, a Member of the WISH/DIP Protein Family in Schizosaccharomyces pombe.

R. Basu, F. Chang; Columbia University, New York, NY

The actin cytoskeleton is dynamically organized into distinct structures that perform specific functions. In fission yeast, actin cables are nucleated by the formin for3p and guide actin-based transport. Actin patches are formed by Arp2/3 complex activated by WASP (wsp1p) and myosin type I (myo1p) and mediate endocytosis. In animal cells, the WISH/DIP proteins have been proposed to a key regulator of actin nucleation, as it may both activate WASP and Arp2/3 activity and inhibit formin activity. Here we characterize the S. pombe orthologue of the WISH/DIP proteins, dip1p. dip1 mutants are viable and exhibit a striking increase in actin cables and a decrease in actin patches. We are testing whether dip1p affects formins and/or WASP. dip1 mutants have an abnormally small number of large actin patches and are defective in endocytosis. In wildtype cells, the appearance of wsp1p at sites of endocytosis is quickly followed by actin assembly and internalization. In dip1 mutant cells, patches of wsp1-YFP reside at the cell cortex more than 10 times longer than in wildtype cells before being internalized. Only a small fraction of wsp1p patches are associated with actin. Dip1p colocalizes with wsp1p in patches on the cortex. Dip1p does not have obvious defects in formin for3p localization or dynamics. These results suggest that S. pombe dip1p functions primarily as an activator of WASP in actin patches for endocytosis.

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Rac1 Regulates Axon Growth through the WAVE-Complex.

S. Tahirovic[1], F. Hellal[1], B. K. Garvalov[1], A. C. Grashoff[2], C. Brakebusch[3], F. Bradke[1]; [1]Axonal Growth and Regeneration Group, Max-Planck Institute of Neurobiology, Martinsried (Munich), Germany, [2]Cardiovascular Research Center, University of Virginia Health System, Charlottesville, VA, [3]BRIC, Biomedical Institute, University of Copenhagen, Copenhagen, Denmark

Neuronal polarization requires distinct changes in the cytoskeleton. While many key regulators have been identified and characterized, relatively little is known about their physiological role in this process. To depict the function of Rac1 in neuronal development, we have generated a conditional knockout mouse in which Rac1 is ablated in the whole brain. Rac1-depletion results in severe defects in cerebellar development. We find that Rac1-deficient cerebellar granule neurons, which do not express any other Rac isoform, fail to extend axons and show impaired neuronal migration. In addition, Rac1 ablation leads to severe defects in lamellipodia formation in growth cones. Analyzing Rac1 effectors, we show that the WAVE-complex is absent from the plasma membrane of knockout growth cones where it normally functions in regulating actin dynamics. We propose that Rac1 recruits the WAVE-complex to enable actin remodelling necessary for neuronal differentiation and cerebellar development.

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Actin Branching Is Affected by Local Bending of the Mother Filament.

V. Risca[1], J. Chia[2], D. Fletcher[1,3]; [1]Biophysics Graduate Group, University of California, Berkeley, Berkeley, CA, [2]The Harker School, San Jose, CA, [3]Bioengineering, University of California, Berkeley, Berkeley, CA

Actin filaments serve as structural elements of the cytoskeleton subject to mechanical forces and provide binding sites for actin-binding proteins (ABPs) at many points on their surface. Structural studies have established that actin filaments can adopt several subtly different twisted structures that are preferentially stabilized by ABPs such as ADF/cofilin or scruin. We sought to investigate whether strain on the actin filament due to bending similarly affects the binding or function of ABPs and chose the Arp2/3 complex, which is involved in the assembly of force-generating actin networks, as our initial target. The Arp2/3 complex nucleates the growth of actin filament branches from mother filaments, making it easy to detect where on the original filament it has bound. To test the effect of bending on branching, rhodamine-phalloidin-stabilized linear actin filaments containing biotinylated monomers were flowed across a streptavidin-coated glass surface. The fluid flow direction was then quickly reversed to promote the binding of filaments to the surface in a bent conformation. The tethered mother filaments were then incubated with actin, Arp2/3 and its activator, and growth of branches was stabilized with green fluorescent phalloidin. We found that branches originating from highly curved sections (radius of curvature < 1.5 um) of actin filaments were more likely to grow from the extended side of the filament (positive strain) than from the compressed side (negative strain), with a statistically significant (P <

0.05) difference from the probability dictated by geometry. We conclude that strain on the filament caused by bending is sufficient to alter actin filament branching, which may play an important role in the organization of actin networks growing under load.

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The Multidomain Oxidoreductase MICAL Is a Specific Orchestrator of F-Actin Dynamics and Links Semaphorins and Plexins to Precise Cytoskeletal Rearrangements.

J. R. Terman, U. Yazdani, R. Hung, J. Yoon, T. Yang; Neuroscience and Pharmacology, The University of Texas Southwestern Medical Center, Dallas, TX

Semaphorins are one of the largest families of axon guidance cues and are now widely studied for their effects on cell movement, morphology, angiogenesis, immunology, and cancer. Semaphorins and their cell surface plexin receptors were initially characterized in part based on their ability to dramatically alter actin cytoskeletal dynamics within motile cells and elongating neuronal processes but the molecules connecting semaphorins and plexins to these cytoskeletal elements are unknown. Recently, we and others have found that highly unusual proteins of the MICAL family of multi-domain oxidoreductase enzymes associate with the cytoplasmic portion of the plexin receptor and mediate both semaphorin-dependent axon guidance and cell morphological changes. Nothing is known, however, regarding the specific role of MICAL in mediating these semaphorin/plexin-dependent morphological changes. We now find that MICAL directly links semaphorins and plexins to specific filamentous actin (F-actin) rearrangements. Our results reveal that MICAL proteins are necessary for normal F-actin bundling during development and require their oxidoreductase domain for this function. Elevating MICAL levels in vivo in an oxidoreductase dependent manner is sufficient to reorganize parallel F-actin bundles into a meshwork of branched actin filaments. MICAL physically associates with filamentous actin and thereby provides the conduit between the semaphorin receptor Plexin, present on the cell surface, and the actin cytoskeleton. Indeed, we find that the MICAL oxidoreductase domain is selectively targeted within cells to control specific actin cytoskeletal rearrangements. These results indicate that MICAL-mediated Redox signaling events are a novel means to orchestrate specific F-actin rearrangements and provide a logic by which semaphorin/plexin-mediated reorganizations of the F-actin cytoskeleton can be achieved in space and time through direct activation of MICAL.

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Acidic Calponin Is Necessary for ERK1/2 Activity and l-Caldesmon Phosphorylation in REF52.2 Fibroblasts.

S. Appel, K. G. Morgan; Boston University, Boston, MA

Calponin is an actin binding, troponin-like molecule, first isolated from chicken gizzard smooth muscle cells where it is involved in regulation of contractility. So far there are three known isoforms, basic, acidic and neutral Calponin, each encoded by a separate gene. Expression of the acidic Calponin isoform is, unlike basic Calponin, not restricted to smooth muscle cells but is expressed in a variety of tissues and cell types, especially in the brain. For basic Calponin it is known that the molecule affects smooth muscle cell contractility by regulation of the PKC/ERK signalling pathway. Here we could show that acidic Calponin co-precipitates together with ERK1/2 and PKCalpha in the cultured rat fibroblast cell line REF52.2. Moreover it colocalises with ERK1/2 and PKCalpha in podosome-like structures and at the cell cortex after PDBu treatment. Knockdown of acidic Calponin expression in these cells results in inhibition of PDBu-mediated ERK1/2 translocation to these structures, whereas PKCalpha targeting is not affected. Both endogenous ERK1/2 activity and phosphorylation of the ERK1/2 substrate l-Caldesmon are blocked by acidic Calponin knockdown, indicating an important role for the acidic Calponin isoform in regulation of ERK1/2 function in REF52.2 cells. Support: NIH grants HL80003, HL86655

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The LIM-only Protein FHL2 Regulates Vascular Smooth Muscle Phenotype.

N. A. Neuman[1,2], G. Lagna[2], A. Hata[1,2]; [1]Biochemistry, Tufts University, Boston, MA, [2]Molecular Cardiology Research Institute, Tufts Medical Center, Boston, MA

Vascular Smooth Muscle Cells (VSMC) are known to have phenotypic plasticity. In response to vascular injury, VSMCs exhibit a change characterized by loss of contractility and abnormal proliferation, migration and matrix secretion. This “synthetic” phenotype plays an active role in repair of the vascular damage. Upon resolution of the injury, the local environmental cues within the vessel return to normal, and VSMCs reacquire their “contractile” phenotype. VSMC phenotype modulation contributes to the pathogenesis of various lung and vascular disorders, including pulmonary arterial hypertension (PAH), atherosclerosis, transplant vasculopathy and restenosis after angioplasty. It is unclear, however, how this phenotypic modulation is regulated. Here we report that the Four-and-a half LIM domains 2 (FHL2) plays a role in the modulation of VSMC phenotype. FHL2 is a component of focal adhesions, binds to the actin cytoskeleton, and acts as transcriptional cofactor in the nucleus. We hypothesized that FHL2 might translate adhesion and structural changes within the VSMC into transcriptional responses, thus affecting its differentiation state or developmental fate. Knockdown of FHL2 by siRNA increased mRNA and protein expression of the contractile apparatus genes smooth muscle α-actin (SMA), SM22α, and calponin. Similarly, overexpression of FHL2 antagonized induction of these genes by the bone morphogenetic proteins (BMPs), the largest subgroup of TGFβs and inducers of the “contractile” phenotype. We have also shown that FHL2 antagonizes the activation of a SMA-luciferase reporter by MRTF-A, a potent transcriptional coactivator of SM-specific genes. Interestingly, chromatin immunoprecipitation experiments indicate that FHL2 does not affect recruitment of serum response factor (SRF) or MRTFA to the SMA promoter upon BMP treatment, however recruitment of RNA polymerase II is severely affected upon association of FHL2 to the SMA promoter. In response to wire injury the carotid arteries of FHL2 homozygous null mice exhibited reduced vascular remodeling compared to wild type mice, demonstrating the essential role of FHL2 in the phenotypic regulation and pathophysiology of VSMC in vivo.

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Chief Cell Specific Expression of Moesin in Gastric Glands.

L. Zhu, B. Zhang, J. Makdisi, J. Hatakeyama, C. Ender, J. Forte; Molecular and Cell Biology, University of California, Berkeley, CA

Chief cells in the gastric glands secrete pepsinogen into the gastric lumen, starting the protein digestion process in the gastrointestinal tract. We previously demonstrated that ezrin is mainly expressed in the acid-secreting parietal cells, but not in other glandular epithelial cells (with the exception of surface epithelial cells). In looking for other members of the ERM family in gastric glands (that is, moesin and radixin), moesin was observed lining the lumen of much of the gastric gland. After eliminating the possibility that moesin is expressed by parietal cells, mucous neck cells and chief cells were the remaining suspects. Because there was a gradient of moesin expression along the gland lumen with an increased level toward the bottom of the glands, we hypothesized that moesin expression is associated with chief cell development from its precursor mucous neck cells. To test this hypothesis, a mucous neck cell probe (lectin GSII) and a chief cell marker (pepsinogen C) were used to stain the glands. Results indicated that GSII positive neck cells at the top of the glands usually did not show moesin expression, but mucous neck cells toward the bottom of the gastric glands and those transitioning cells (from mucous neck cells to chief cells) express moesin. The level of moesin expression continues to increase toward the bottom of the glands, and reaches a plateau in the middle of the glands. The pepsinogen expression level also increases toward the bottom of the glands, with a similar gradient expression pattern as moesin, although the subcellular localization for pepsinogen C and moesin is different (pepsinogen C is located in the rER and zymogenic granules; while moesin is exclusively expressed on the apical membrane). This is a clear demonstration of distinctive cellular expression of two ERM family members. The increased expression of moesin from mucous neck cells to chief cells, and its apical membrane localization, provide the first evidence that moesin is involved in the cell biology of chief cells.

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Phosphoisoforms of Ezrin in Gastric Glands and Proximal Tubules.

L. Zhu, K. Poon, B. Zhang, M. Starr, C. Ender, J. Forte; Molecular and Cell Biology, University of California, Berkeley, CA

Early studies have indicated a receptor activated increase in phosphorylation of the membrane cytoskeletal linker ezrin in both model cell lines and in situ tissue, especially on residue T567 (T567P). In this work we sought to identify the various phosphoisoforms of ezrin in isolated gastric glands and renal proximal tubules in the context of different physiological conditions. Up to 8 different 80 kDa ezrin phosphoisophorms (numbered spot 1- 8) have been detected using 2D electrophoresis; spot 1 is the most basic at with a pI ~6.7 with the more acidic isoforms ~0.05 pH units apart. For control glands (no stimulants added) spot 1 was the most abundant, spot 2 was less abundant, and spot 3 (and higher) represented less than 20% of the total ezrin. Spots 1 & 2 were devoid of T567P, whereas more acidic spots always included T567P. Stimulation of gastric glands with secretagogues shifted ezrin to acidic isoforms. Inhibition of rotein phosphatase with calyculin A (CLA) produced a complete loss of spots 1 & 2, shifting all spots to the more acidic isoforms. Comparative studies revealed that freshly isolated proximal tubules had considerably more ezrin phosphorylated at T567, thus more acidic isoforms, than control gastric glands. Accordingly, CLA produced only a modest shift toward acidic isoforms, whereas, hypoxia shifted the isoforms primarily to spots 1 and 2. These data demonstrate (i) T567 as a functionally important site (consistent with many studies), (ii) there must be several additional phosphorylation sites on ezrin, and (iii) spots 1 & 2 are certainly not phosphorylated on T567, thus something else must account for their different pI's. We are performing similar 2D electrophoresis to see if phosphorylation on tyrosine or serine residues accounts for the higher order spots or the difference between spots 1 & 2, and how these phosphorylation events correlate with different physiological conditions in the gastric parietal cells.

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Hierarchal Autoregulation of Zyxin-VASP Complexes.

J. Grange, M. Ascione, D. Boothe, B. Fillmore, J. Moody, M. Hansen; Physiology and Developmental Biology, Brigham Young University, Provo, UT

Cell adhesion sites are characterized by actin-membrane linkages and dynamic actin rearrangements. VASP is an actin regulator that functions at adhesion sites. Its localization to adhesion is mediated by proteins containing proline-rich motifs that bind VASP's EVH1 domain. Zyxin bears an array of four proline-rich VASP binding motifs and is required for VASP localization to focal adhesions. Genetic deletion is associated with increased cell motility and cell-substrate adhesion. While zyxin may simply recruit VASP to cellular sites, evidence also suggests a role for zyxin's LIM domains in regulating VASP activity. However, a molecular basis for how zyxin might alter VASP function has not been addressed. Here, in vitro biochemical assays are used to dissect zyxin-VASP interactions and define their effect on actin remodeling at the molecular level. We find that zyxin is controlled by two distinct autoregulatory mechanisms, both mediated by the LIM domains. The LIM domains mediate a head-tail interaction within zyxin that prevents VASP binding to zyxin's proline-rich motifs. This mechanism is selective for PKA-phosphorylated forms of VASP; unphosphorylated zyxin bypasses this first autoregulatory mechanism, a result of a direct interaction between VASP and zyxin's LIM domain region. Though the LIM-VASP interaction alone has no effect on VASP's actin regulatory activities, it provides a second autoregulatory comtrol to zyxin-VASP complexes. When zyxin binds VASP through both the LIM domains and the proline-rich motifs, VASP is prevented from participating in control of actin remodeling. This two-tiered autoregulatory system allows cells to control zyxin-VASP complex assembly and function. Cellular controls for activating zyxin-VASP complexes at both levels are explored.

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Interaction of Yeast Profilin with the 167-170 Loop of Yeast Actin.

M. McKane, K. Wen, P. Rubenstein; Biochemistry, University of Iowa College of Medicine, Iowa City, IA

Profilin is believed to bind to the barbed end of G-actin across subdomains 1 and 3 causing a conformation change, possibly involving the actin 167-170 loop, that leads to the acceleration of exchange of the actin-bound nucleotide. This acceleration may be due to an interaction between an Arg in profilin, R81 in yeast profilin (YPF), and actin residue 167. The resulting steric crowding would force open the nucleotide binding cleft in actin leading to enhanced exchange rates. Here we examined the crowding hypothesis using a mutant yeast actin, A167E, in which E, found in muscle actin, is substituted for A found in yeast actin in order to increase potential crowding. A167E actin causes profound growth defects in yeast but appears to polymerize normally in vitro. Both WT and A167E Ca-actins have similar exchange rates for bound-εATP, (36 sec, T1/2). WT YPF accelerates the exchange rate for WT actin 3-fold but that of A167E actin 7-fold in agreement with our

hypothesis. To reverse the crowding caused by 167E, we made the R81A YPF in E. coli. However, no binding to actin was detected, and R81A profilin yeast cells with either WT or 167E actin could not be obtained. Another important interaction with actin is believed to involve neighboring residue Y79 of yeast profilin. We thus altered Y79 to S to reduce crowding while maintaining the OH group. Y79S profilin rescues the growth phenotype caused by A167E actin. Both WT and Y79S YPF bind to Ca-A167E with similar affinities based on a fluorescence assay. However, this mutant profilin only accelerates nucleotide exchange 2-fold. With Mg-actin, which has an exchange T1/2 of about 10 sec., both WT and Y79S YPF cause a 2-fold acceleration of exchange rate. The data suggest that with Ca-actin, increasing the crowding between the actin 167-170 loop and profilin accelerates actin-bound ATP release, but with Mg-actin, a more dynamic structure, this crowding effect is decreased. A lack of correlation between in vitro nucleotide exchange data and the dramatic growth rescue seen with Y79S profilin in A167E actin-containing cells implies that the major biological function of profilin/actin binding may not be due to enhancement of actin-bound nucleotide exchange.

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The Dictyostelium Pod-1 Homolog Crn7 Is a Coronin-like Protein with Roles in Actin-driven Processes and Infection with Legionella pneumophila.

M. Shina[1], C. Unal[2], A. Mueller-Taubenberger[3], M. Steinert[2], A. A. Noegel[1]; [1]Biochemisty I, Medical Faculty, Cologne, Germany, [2]Institute for Molecular Infection Biology, University of Wuerzburg, Wuerzburg, Germany, [3]Ludwig Maximilians University Munich, Institute for Cell Biology and Center for Integrated Protein Science (CIPSM),, Munich, Germany

Coronins are multifunctional proteins containing WD40 repeat domains that form a b-propeller structure predestined for protein-protein interactions. Here we describe corB, one of two genes in Dictyostelium discoideum that code for coronin proteins. corB encodes a 962 amino acid protein, Crn7, that, in contrast to the corA product coronin, contains two complete WD repeat domains thus resembling Crn7, the long coronin in man, and Pod-1 of Drosophila melanogaster and Caenorhabditis elegans. D. discoideum Crn7 shares highest homology with its human counterpart and harbors also a proline-serine-threonine (PST) rich domain that is unique for D. discoideum and human Crn7 and separates the two WD repeat domains. D. discoideum Crn7 is present in the cytosol as well as in cytoskeletal and membrane fractions. During phago- and macropinocytosis Crn7 accumulates at phago- and pinocytic cups. Strains lacking Crn7 have severe defects in actin driven processes like phagocytosis, and development. Further, Crn7 affects the infection process by the pathogen Legionella pneumophila where it functions differently from coronin. Our results suggest that Crn7 has specific roles in F-actin-mediated cytoskeletal rearrangements and that it is an important factor in protection against bacterial infection.

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Drebrin Influences Adhesion, Morphology and Cell-Cell Contact Formation in Human Endothelial Cells.

V. van Vliet[1], A. Osiak[1], W. Peitsch[1], M. Sixt[2], A. Schröder[3], B. Ebbing[4], B. Böhlig[1], S. Linder[5]; [1]Institute for Prevention of Cardiovascular Diseases, LMU, Munich, Germany, [2]Medizinische Fakultät Mannheim, University of Heidelberg, Mannheim, Germany, [3]Max von Pettenkofer Institut, LMU, Munich, Germany, [4]Institut für Zellbiologie, LMU, Munich, Germany, [5]Max Planck Institute for Biochemistry, Martinsried, Germany

Drebrin is an f-actin binding protein originally identified in neuronal cells, but is also expressed in diverse non-neuronal cells such as kidney or human umbilical vein endothelial cells (HUVEC). Drebrin is involved in modification of actin cytoskeletal organization and affects cell morphology. However, the molecular mechanisms of these phenomena are mostly unclear. Here, we investigate the role of drebrin (isoform E2) in endothelial adhesion, spreading, cell cortex remodeling and cell-cell contact formation. During adhesion and spreading, drebrin becomes highly enriched at the cell periphery and also in highly dynamic actin-rich adhesion structures displaying podosomal characteristics. Downregulation of drebrin by siRNA, as well as microinjection/overexpression of various drebrin domains disrupts these structures. Overexpression of drebrin does not influence the spreading rate of HUVEC, but leads to dramatical remodeling of the cell cortex and of peripheral adhesions. These phenomena are coupled with local defects in de-adhesion of protrusions and altered Rho/Rho kinase activity. Knock down of drebrin by siRNA/shRNA i) does not substantially reduce the ability of cells for wound healing, but ii) leads to altered cell-cell contact formation, most notably a dislocalization of VE-cadherin, and iii) influences the ability of HUVEC for tube formation in Matrigel assays. These data indicate that drebrin has an important modulatory role on actin cytoskeletal function in multiple phases of endothelial physiology.

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C. elegans Gelsolin-like Protein 1 (GSNL-1) Is an Actin Filament Severing Protein with Four Gelsolin-like Repeats.

T. Klaavuniemi, S. Yamashiro, K. Ono, S. Ono; Pathology, Emory University, Atlanta, GA

The gelsolin family of proteins is a major class of actin regulatory proteins that sever, cap, and nucleate actin filaments in a calcium-dependent manner and are involved in various cellular processes. Typically, gelsolin-related proteins have three or six repeats of gelsolin-like (G) domain, and each domain plays a distinct role in severing, capping, and nucleation. The *Caenorhabditis elegans* gelsolin-like protein-1 (*gsnl-1*) gene encodes an unconventional gelsolin-related protein with four G domains. We purified bacterially expressed GSNL-1 and characterized its interaction with actin in vitro. GSNL-1 severed actin filaments and capped the barbed end in a calcium-dependent manner. The severing activity of GSNL-1 was weaker than that of bovine serum gelsolin. However, unlike gelsolin, GSNL-1 remained bound to the side of F-actin with a sub-micromolar affinity and did not nucleate actin polymerization although it bound to G-actin with high affinity at a 1:1 molar ratio. These results indicate that GSNL-1 is a novel member of the gelsolin family of actin regulatory proteins. Previous reports by others have shown that GSNL-1 is predominantly expressed in the body wall muscle. However, a *gsnl-1*-knockout worm had normal organization of striated myofibrils and did not exhibit a defect in contractile activity of the body wall muscle. In contrast, overexpression of GFP-tagged GSNL-1 in the body wall muscle resulted in paralysis. When GFP-GSNL-1 is moderately expressed, it localized to the myofibrils and the cell-cell junctions, suggesting that actin severing activity of GSNL-1 is normally suppressed by an unknown mechanism. Taken together, GSNL-1 is a candidate regulator of actin filament organization in muscle, and *C. elegans* will be an excellent model to investigate cellular functions of gelsolin-related proteins.

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Localization and Transport of Profilin mRNA in Cultured Cells.

A. E. Johnsson, R. Karlsson; Dept. of Cell Biology, WGI, Stockholm University, Stockholm, Sweden

The leading edge of migrating cells advances over the substratum by actin polymerization-driven formation of protruding lamellipodia and filopodia (Koestler *et al*. (2008) *Nat Cell Biol* 10:306-13). Different actin filament-forming protein machineries like Ena/VASP and formins operate as processive motors at the tip of these structures where they incorporate actin into growing filaments by using profilin:actin as source of unpolymerized actin (e.g. Grenklo *et al*. (2003) *EMBO Reps* 4:523-9; Lindberg *et al*. (2008) *Sem in Cancer Biol* 18:2-11). Monomer incorporation into the filaments is rapid and diffusion of profilin:actin is not sufficient for supplying enough actin monomers to support the extensive polymerization occuring at the leading edge (Lai *et al*. (2008) *EMBO J* 29:982-92). It has been reported that mRNA:s encoding β-actin and components of the Arp2/3 complex, which is involved in actin nucleation are actively transported to regions of the cell where massive actin polymerization takes place (Mingle *et al*. (2005) *J Cell Sci* 118:2425-33). We have studied the distribution of profilin mRNA, using fluorescent *in situ* hybridisation (FISH) in mouse embryonic fibroblasts (MEF-cells), and have observed that profilin and β-actin mRNA:s both localize to the dorsal ruffles typically formed on these cells after serum stimulation. Furthermore, our observations suggest that the transport of profilin mRNA is dependent on the microtubule system.

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SmAV Is an ERK Scaffolding Protein.

S. S. Gangopadhyay[1], E. Kengni[1], C. Gallant[1], H. Kim[1], P. Leavis[2], J. DeGnore[3], S. Appel[1], K. G. Morgan[1]; [1]Boston University, Boston, MA, [2]Boston Biomedical Research Institute, Watertown, MA, [3]Tufts University School of Medicine, Boston, MA

ERK (extracellular signal-regulated kinase) influences a number of pathways in all cells, but how ERK's activities are segregated between different pathways has not been entirely clear. Using immunoprecipitation and pull down experiments with domain-specific recombinant fragments we show that SmAV binds ERK and members of the ERK signaling cascade in a domain-specific, stimulus-dependent and pathway-specific manner. MEK binds specifically to the first 445 residues of SmAV. B-Raf, an upstream regulator of MEK constitutively interacts with both residues 1-445 and 446-1250. Both ERK and 14-3-3 bind to both fragments, but in a stimulus-specific manner. Phosphorylated ERK is associated only with residues 1-445. An ERK phosphorylation site was determined by mass spectrometry to reside at Ser132. A phospho-antibody raised to this site shows that the site is phosphorylated during alpha agonist-mediated ERK activation in smooth muscle tissue. Phosphorylation of SmAV by ERK decreases the association of phospho-ERK with SmAV. These results, combined with previous observations indicate that SmAV serves as a new ERK scaffolding protein and provide a mechanism for regulation of ERK binding, activation and release from the signaling complex.

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Regulation of Somitogenesis by Rho GTPases during X. laevis Development.

M. E. Greene; Biology, San Francisco State University, San Francisco, CA

Congenital defects such as scoliosis and other spinal deformities result from a disruption in somitogenesis, a developmental process by which somites are formed. The formation of somites is crucial since these structures will give rise to the vertebrae, muscle, and dermis in the adult organism. Prior work from our lab has characterized the different cell behaviors that occur during somitogenesis within Xenopus laevis. These behaviors involve actin-based filopodial and lamelopodial protrusions and appear to be regulated by RhoA and Rac1, members of the Rho family of GTP--binding proteins. While the cell morphology has been characterized, the molecular mechanisms controlling these behaviors are not well understood. To investigate the role these GTPases play during somitogenesis, X. laevis embryos were micro-injected with RNA that impairs the function of the wild-type RhoA and Rac1 proteins. Confocal images of mutant embryos reveal that the cells in the paraxial mesoderm do not undergo proper segmentation and rotation. Although somites do form, they consist of myotome fibers that are mis-aligned and disorganized. These results provide evidence that the actin regulatory proteins, Rho A and Rac1, are associated with the changes in cell behavior that leads to the normal development of somites. This study is significant as it will make an important contribution to our understanding of the regulatory molecules underlying the cell behaviors associated with vertebrate segmentation.

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Characterization of Keratocyte Motility in BAF53a Heterozygote Zebrafish.

A. Dennison, C. Wills, E. Hull; Biomedical Sciences, Midwestern University, Glendale, AZ

BAF53a is an actin related protein (ARP) that binds actin filaments in an IP3 dependant fashion and is a member of an SWI/SNF type chromatin remodeling complex. Keratocytes cultured from zebrafish heterozygote for an insertion mutation in the BAF53a gene have reduced motility. In explant cultures, cell sheets grown from BAF53a heterozygotes cover less area when compared to cultures from wild-type animals (wild type 0.059 mm2 and the mutant 0.028 mm2, Mann-Whitney test U = 2008, p= 4.3 x 10-4). To control for variation in the amount of tissue initially cultured, a single linear dimension at the scale-cell sheet boundary was used to indicate the amount of tissue in the initial explant culture. As there was no statistical significance (Mann-Whitney test U= 2584, p= 0.13) between BAF53a and wild-type cultures, we concluded that differences in initial tissue amount did not explain the decrease in spread area. Consistent with these results, the motility of individual migrating keratocytes cultured from BAF53a heterozygotes is greatly reduced, as these cells are unable to maintain a stable lamella. Although BAF53a protein levels are reduced in heterozygotes, it is unclear whether reduction in the actin binding or SWI/SNF chromatin remodeling activity is responsible for the observed phenotype.

357/B301

Developmental Expression Profile of Annexin 5a in the Inner Ear of the Mouse.

M. Drummond[1,3], I. Belyantseva[2], M. Zhu[3], K. Friderici[1,3]; [1]Genetics Program, Michigan State University, East Lansing, MI, [2]Laboratory of Molecular Genetics, NIDCD/NIH, Rockville, MD, [3]Microbiology and Molecular Genetics, Michigan State Universtiy, East Lansing, MI

Non-syndromic sensorineural hearing loss is one of the most common communication disorders. We reported previously that missense mutations in γ-actin gene (ACTG1) result in progressive sensorineural hearing loss. It is known that γ and β-actin are cytoplasmic actins expressed in all mammalian cell types, and γ-actin is a predominant actin in sensory hair cells of the inner ear. Annexin 5a (ANXA5) is the only protein known to interact preferentially with γ-actin and not β-actin. Massively Parallel Signature Sequencing data indicate that Anxa5 is expressed at very high level in the mouse inner ear relative to its expression in other organs and tissues. It was reported that Annexin 5a may play a role in the reorganization of the actin cytoskeleton, leading us to postulate that its interaction with γ-actin may be important in maintenance of the cytoskeleton in the organ of Corti. We investigated the localization and developmental expression profile of Annexin 5a in the mouse inner ear. Using a GST-pulldown assay, we have confirmed that ANXA5 interacts exclusively with the γ isoform of cytoplasmic actin. To examine the expression profile of ANXA5 in the inner ear, whole-mounts of the organ of Corti and vestibular end-organs from P0, P5, P7, P12, P21, P30 and P60 mice were stained with an anti-ANXA5 antibody. The expression profile data demonstrates changes in localization and expression of ANXA5 during development in the mouse inner ear. Via a gene gun mediated transfection, we examined targeting and localization of ANXA5 in postnatal cochlear explants using a C-terminal GFP-fusion to Anxa5. We found that GFP-ANXA5 localizes to the cuticular plate of hair cells and vesicle-like structures of the supporting cells in postnatal organ of Corti explants. Our data suggest that ANXA5 may be delivered via vesicular transport to incorporate into the membrane of hair cell cuticular plate and stereocilia.

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A Novel Conserved Protein Involved in Bacterial Cell Morphogenesis.

A. Alyahya[1], R. Alexander[1], T. Emonet[1], C. Jacobs-Wagner[1,2,3]; [1]Department of Molecular,Cellular and Developmental Biology, Yale University, New Haven, CT, [2]Section of Microbial Pathogenesis, Yale University, New Haven, CT, [3]HHMI, Yale University, New Haven, CT

For millions of years, bacteria have existed in the environment in various shapes and sizes. Studying the mechanisms that govern this morphological diversity is fundamentally important to the understanding of how organisms translate genetic information into higher order structures that will then be propagated reproducibly into their progeny. More specifically, in order to obtain a specific morphology, bacteria must regulate cell growth accurately at a spatial and temporal level. In this study, we used the Gram-negative curved rod-shaped organism, *Caulobacter crescentus*, to search for novel genes implicated in cell morphogenesis via a visual genetic screen for cell shape mutants. We show that a novel conserved gene, tentatively named *jabA*, is essential for growth and survival. JabA functions by localizing to areas of cell growth. Depletion of JabA results in cells that become progressively larger and eventually lyse. JabA interacts with the prokaryotic actin homolog, MreB,that has been implicated in bacterial rod-shape determination. In the absence of correct MreB localization, JabA mislocalizes. Bioinformatic analysis suggests that JabA can be found across the bacterial kingdom and we thus extended our study into the gram-negative bacterium *Escherichia coli* that has been shown to possess a different mode of growth to that of *C. crescentus*. A deletion of $jabA_{Ec}$ *(yfgA)* in *E. coli* results in cells that are largely misshapen and rounder relative to wild-type. Consistently, $JabA_{Ec}$ exhibits a helical pattern of localization that co-localizes with MreB helices, suggesting that the two proteins interact in regulating cell morphology in *E. coli*. These studies suggest that JabA is a novel morphogenetic protein that plays an important role in topological control of cell growth in bacteria.

359/B303

An SiRNA-based Screen for Motor Proteins That Control Lysosomal Trafficking and Integrity in Cancer Cells.

L. Groth-Pedersen, J. Nylandsted, M. Jäättelä; Apoptosis Department, Danish Cancer Society, Copenhagen, Denmark

Tumor invasion and metastasis are associated with altered lysosomal trafficking and increased expression of the lysosomal proteases cathepsins. These changes may be necessary for cancer progression, but emerging experimental evidence suggests that they also render tumor cells more sensitive to lysosomal membrane permeabilization and subsequent cathepsin-mediated cell death. Thus, the lysosomal compartment is likely to be a good target for cancer therapy. The aim of this study is to identify proteins that control lysosomal trafficking and integrity in cancer cells in order to find new targets for cancer therapy. For that purpose we have designed an siRNA library in triplicate that target 146 known motor proteins (e.g. kinesins, dyneins and myosins). A screen for cell death revealed six motor proteins whose depletion by a minimum of two independent siRNAs results in over 40% reduction in the survival of MCF-7 breast cancer cells. Another screen for lysosomal localization revealed that depletion of Tropomyosin2, an actin-binding protein that was also found to induce cell death in the first screen, induced a dramatic lysosomal relocalization to the periphery of the cell. We are presently evaluating the screen hits, with special focus on the involvement of lysosomal membrane permeabilization in the cell death processes induced by depletion of the motor proteins.

360/B304

Phosphorylation of Vasodilator Stimulated Phosphoprotein (VASP) Is Affected by Ventilation Mode In the Preterm Lamb Model of BPD.

K. Albertine, M. Yoshigi, L. Dong, M. Dahl, J. Alvord, C. Callaway, R. McKnight, L. Moyer-Mileur, D. Null, R. Lane; Pediatrics, University of Utah, Salt Lake City, UT

Background: While ventilator-induced mechanical stretch of the immature lung contributes to the pathogenesis of bronchopulmonary dysplasia (BPD), the mechanisms remain unknown. We recently identified VASP as a unique molecule that rapidly responds to mechanical stretch, regulates remodeling of the actin cytoskeleton, and adapts to mechanical stretch by changing its phosphorylation status. Objective: We hypothesized that mechanical ventilation-induced stretch of the premature lamb lung alters the phosphorylation state of VASP. Methods: To evaluate developmental changes in VASP in the lamb lung, we compared 4 developmental age groups: fetus 132d gestation, fetus 136d gestation, term newborn 1d (24h), and term newborn 3d (72h) (term~150d). To evaluate responses to ventilation mode, we compared 6 groups of ventilated lambs: preterm, mechanical ventilation for 1d or 3d, or high-frequency nasal ventilation (HFNV) for 1d or 3d, as well as term lambs for 1d or 3d (n=4/group). We quantified VASP phosphorylation in lung tissue homogenates by densitometry of each doublet band to calculate phospho-VASP to total-VASP (pVASP/tVASP) ratio. Immunohistochemistry localized pVASP and tVASP. Results: During late-

gestation, tVASP protein abundance and pVASP/tVASP protein ratio increased from 132d gestation to 136 d gestation, and subsequently decreased by birth at term gestation. MV of term lambs for 1 or 3d did not affect tVASP abundance or pVASP/tVASP protein ratio. On the other hand, MV of preterm lambs for 1d decreased tVASP and increased pVASP/tVASP protein ratio. By comparison, HFNV of preterm lambs decreased both tVASP abundance and pVASP/tVASP protein ratio. VASP was localized to airway epithelial cells, vascular smooth muscle cells, and lung parenchymal cells. Conclusion: Ventilation mode alters the phosphorylation of VASP protein in chronically ventilated preterm lambs. Because HFNV of preterm lambs results in less pVASP than MV, less stretch-induced cell signaling may contribute to the normal alveolar formation in preterm lambs when HFNV is used. (HL62875, HL56401, EB04443, CHRC)

Actin (361-374)

361/B305

In Vivo Dynamics of a Unique Family of Actin Homologs Involved in Organelle Positioning in Bacteria.

M. Byrne, A. Komeili; Plant and Microbial Biology, UC Berkeley, Berkeley, CA

Recent advances in bacterial cell biology have revealed the existence of cytoskeletal elements in bacteria, including a number of actin homologs that perform essential cellular functions. One family of bacterial actin-like proteins called the MamK family has been found to be important in the positioning of subcellular compartments. We are interested in understanding the in vivo dynamics of MamK filaments, their regulation, and eventually, the mechanisms of their cellular function. MamK is found in magnetotactic bacteria and is necessary for the organization of an intracellular chain of membrane-bound magnetic crystals, called the magnetosome chain. By fluorescence light microscopy, MamK-GFP forms a linear filament that extends pole to pole in Magnetospirillum magneticum sp. AMB-1. We used fluorescence recovery after photobleaching (FRAP) analysis to determine that MamK-GFP has a recovery half-time of about 10 minutes with no obvious polarity of subunit exchange. A point mutation in the ATP-binding pocket (D161A) inhibits filament recovery, which suggests ATP hydrolysis is necessary for subunit exchange as in other actin homologs. Also, MamK-GFP expressed in a strain of AMB-1 lacking the magnetosome island (MAI), which is a 98 kb genomic region necessary for magnetosome formation, forms short filaments with little or no subunit exchange, suggesting that one or more proteins encoded in the MAI help stimulate turnover of the filament and regulate overall filament length. To further explore the polymerization dynamics of MamK, we made a series of mutations in putative surface-exposed residues. We have identified mutants that form filaments of aberrant length and have recovery half-times that are faster and slower than wild-type MamK. These mutants will help us to determine interaction surfaces between MamK subunits and to investigate sites of protein interaction important for regulation of MamK filaments or for recruitment of other proteins to the magnetosome chain. Beyond their relevance to magnetotactic bacteria these results are likely to provide general insights into the dynamics and evolution of actin-like proteins.

362/B306

Assembly Properties of the Bacterial Actin, MreB.

G. J. Bean[1], J. Mayer[2], L. Hazelwood[3], D. Hanein[3], K. Amann[1,2]; [1]Zoology / Molecular Biology, UW-Madison, Madison, WI, [2]Cell and Molecular Biology Program, University of Wisconsin-Madison, Madison, WI, [3]Burnham Institute, La Jolla, CA

We have characterized biochemically and structurally the assembly properties of the bacterial actin, MreB, from *Thermotoga maritima* and *Bacillus subtilis*. Biochemically, the *B. subtilis* molecule behaves similarly to *T. maritima* MreB, with several physiologically relevant exceptions. Most significantly, *B. subtilis* MreB assembly is independent of the bound nucleotide. As observed by fluorescence and electron microscopy, both molecules assemble into large polymeric structures roughly analogous to bundled eukaryotic actin filaments, though built from single protofilaments, not two-start helical filaments. We found that MreB assembles from monomers to polymers through a nucleation pathway completely different from that of eukaryotic actins, characterized by short, flexible, linear, oligomeric intermediates rather than the classical trimer. We have also characterized the interaction between MreB and the drug A22. We found that A22 does not directly disassemble MreB filaments but instead lowers the critical concentration to a value corresponding to that of ADP-bound MreB. We will discuss the cellular and physiological implications of these findings.

363/B307

The Actin Cytoskeleton of Giardia intestinalis.

A. R. Paredez[1], S. C. Dawson[2], Z. Cande[1]; [1]MCB, UC Berkeley, Berkeley, CA, [2]Microbiology, UC Davis, Davis, CA

Giardia intestinalis is a flagellated intestinal parasite that causes diarrheal disease in a wide variety of hosts including over 100 million people each year. A diplomonad, it is thought to belong to the earliest diverging eukaryotic lineage, and is much more divergent from yeast than yeast is from man. Thus, *Giardia* is a valuable model organism for gaining key insights into early eukaryotic cell evolution. *Giardia* has a complex microtubule cytoskeleton including basal bodies and axonemes and novel structures such as the ventral disc, an organelle involved in parasite attachment to the intestinal wall of the host. Although the majority of the components of the microtubule cytoskeleton can be identified in the giardial genome, no members of the canonical actin cytoskeleton can be identified except for actin itself (58% identity on average), and three divergent actin related proteins (ARPs). Missing from the giardial genome are myosin motors, Arp2/3 and other proteins needed for actin nucleation, proteins that regulate actin polymerization such as profilin, and actin cross linking proteins. Our objective is to characterize the divergent giardial actin cytoskeleton which we expect to provide a unique perspective on actin cell biology and evolution. Phylogenetic analysis reveals that all three giardial ARPs cluster with either Arp8 or Arp9 and GFP fusions to these ARPs localize to the nuclei suggesting they function in chromatin remodeling and not filament formation. We have generated an antibody against giardial actin that interestingly labels specific actin structures of unknown function as well as the ventral disk, nuclei, and axonemes. Expression and purification of giardial actin using a baculovirus expression system in hopes of characterizing the biochemical properties of this divergent actin are underway.

364/B308

Actin Filament Organization and Remodeling by Dynamin2 GTPase.

O. L. Mooren[1,2], T. Kotova[1], D. Schafer[1]; [1]Biology, University of Virginia, Charlottesville, VA, [2]Cell Biology, Washington University, St. Louis, MO

Dynamin2 GTPase activity regulates actin filament turnover in cells but the mechanisms by which dynamin2 influences actin filaments are unknown. Cortactin binds to dynamin2, which could provide a biochemical link between dynamin2 and actin filaments. We observed the effects of dynamin2 on actin filament organization and stability using biochemical and microscopic assays. Dynamin2 bundles actin filaments nucleated by Arp2/3 complex and cortactin. Dynamin2-induced filament bundles observed by transmission electron microscopy (TEM) were highly ordered, with a repeating transverse striated pattern and organizational dependence on nucleotide state. Dynamic changes in dynamin2-induced bundled filaments following GTP addition were observed by total internal reflection fluorescence microscopy (TIRFM), with filament ends splaying out from bundles or unraveling. Since actin depolymerization factors function, in part, by severing actin filaments, we tested the hypothesis that dynamin2 GTPase activity may promote filament disassembly by a similar mechanism. Actin filaments formed in the presence of Arp2/3 complex, cortactin, and dyn2 had an increase in the number of polymerizable ends following GTP addition, as assessed using pyrene-labeled actin assays, suggesting that dynamin2 GTPase activity either creates or exposes new filament ends. Additionally, dynamin2-induced bundles were incubated with an actin filament severing protein, cofilin, and the number of filament ends were assessed by pyrene-actin polymerization. Cofilin had a minimal effect on dynamin-induced bundles in the absence of nucleotide. GTP, but not GTPγS, promoted severing of filaments by cofilin. Thus, our findings suggest that dynamin GTPase activity promotes actin filament turnover via a mechanism that may involve breakage of actin filaments or by altering access of cofilin to filaments.

365/B309

WASH Proteins Integrate Vesicular F-actin Dynamics with Microtubules during Endocytic Trafficking.

T. S. Gomez, D. D. Billadeau; Department of Biochemistry and Molecular Biology, College of Medicine, Mayo Clinic, Rochester, MN

The Arp2/3 complex is required for the endocytic process, yet the precise Arp2/3-stimulating factors that orchestrate the diverse hierarchal steps during receptor trafficking and sorting remain ill-defined. WASH (Wiskott-Aldrich Syndrome Protein and SCAR Homolog) was recently discovered as a widely expressed and polymorphic Arp2/3 complex activator, which has undergone subtelomeric duplication during primate evolution. Humans display the highest WASH copy numbers along with variable WASH repertoires. Here we examined the functional role of human WASH proteins, and found via immunofluorescence studies that endogenous WASH distinctly co-localized with early endosomes in various cell types. Moreover, WASH localized with vesicular F-actin and inducibly interacted directly with microtubules. Although WASH dynamically associated with newly internalizing epidermal growth factor receptors and T cell receptors (TCR), siRNA-mediated depletion of WASH did not impact cell surface receptor clearance following stimulation. However, TCR stability and surface expression were diminished by loss of WASH proteins, suggesting a role for WASH in receptor sorting/trafficking. Therefore, WASH may be a critical regulator of the early endocytic compartment, functioning at the interface between endosomes, microtubules and the F-actin cytoskeleton.

366/B310

Synaptopodin Regulates Actin Dynamics by Blocking the Proteasomal Degradation of Nck1.

E. Lopez-Rivera, C. Faul, P. Mundel; Department of Medicine, Miami Institute of Renal Medicine, Miami, FL

Podocytes of the renal glomerulus are unique cells with a complex cellular organization consisting of a cell body, major processes and foot processes. The dynamic regulation of the podocyte actin cytoskeleton is paramount for proper kidney filter function and mutations affecting several podocyte proteins lead to the rearrangement of the actin cytoskeleton, disruption of the filtration barrier and renal disease. The ubiquitously expressed SH2/SH3 adapter proteins Nck1 and Nck2 contribute to the regulation of podocyte actin dynamics by linking phospho-nephrin to N-WASP:ARP2/3 mediated actin polymerization. Here we show that in podocytes Nck1 but not Nck2 steady-state proteins levels are regulated by Cbl mediated ubiquitination and subsequent proteasomal degradation of Nck1. The inactivation of an evolutionary conserved lysine residue (K178R) that is present in Nck1 but not in Nck2, reduces Cbl binding and proteasomal degradation of Nck1. Synaptopodin, a critical regulator of podocyte actin dynamics, directly binds to Nck1 and competes with Cbl for Nck1 binding, thereby preventing the ubiquitination of Nck1. In summary, these results demonstrate a role for synaptopodin as a regulator of Nck1 proteasomal degradation. Together with the recently identified inhibition of Smurf1 mediated ubiquitination of RhoA, these results unveil synaptopodin as a comprenhensive inhibitor of E3 ubiquitin ligase controlled actin signaling.

367/B311

Differential Processing of Beta and Gamma Actin Confers Differences in Posttranslational Modifications In Vivo.

F. Zhang, A. Kashina; Animal Biology, University of Pennsylvania, Philadelphia, PA

Actin is a major component of the cytoskeletal network. Non-muscle cells contain two major actin isoforms, beta and gamma, that are highly homologous in their primary sequences (>98% identity) but are more different at the nucleotide level (88% identity). Beta actin is posttranslationally arginylated on the 3rd Asp and this modification is a key modulator of the actin network. Remarkably, gamma-actin is never found arginylated in vivo on the same site despite its high similarity to beta actin. To investigate how the arginylation machinery distinguishes between these two isforms, we tested the metabolic fate of exogenously expressed permanently arginylated beta and gamma actin constructs in vivo. Our result shows that arginylated gamma actin is significantly less stable at the protein level compared to arginylated beta actin, and undergoes rapid proteasome-dependent degradation in cells. Since the two actin isoforms are highly identical in their amino acid composition, such a dramatic difference in the degradation rate is unlikely to originate from the primary structure, but rather arises at the mRNA level through differential translation dynamics. To test this hypothesis, we switched the coding sequences of beta and gamma actin, to obtain a 'converted' gamma actin encoded by a beta-like nucleotide sequence and vice versa for a 'converted' beta actin. 'Converted' arginylated gamma-actin was much more stable than the arginylated wild type gamma or the 'converted' beta actin, confirming that the differences in the processing and stability of the two actin isoforms indeed originate at the mRNA level. This the first demonstration of the

differential posttranslational processing of the two nonmuscle actin isoforms that arises at the nucleotide level. Ongoing experiments address the translation dynamics/speed of the two actin isoforms and the role of Lys residue proximal to the N-terminus in the degradation process. Our data suggest a plausible explanation for the lack of arginylation of gamma-actin in vivo and a novel mechanism of a degradation event coupled with translation/folding dynamics that confers different properties and functions to the nonmuscle actin isoforms.

368/B312

Actin and Myosin Involvement in Sex-Chromosome Anaphase Movements in Crane-Fly Spermatocytes.

J. Su, A. Forer; Biology, York University, North York, ON, Canada

In crane-fly spermatocytes, autosomes move poleward during anaphase while the two univalent sex chromosomes remain at the equator. The sex chromosomes segregate only after the autosomes reach the poles. Each sex chromosome has kinetochore fibres connected to each pole: one fibre shortens and one fibre elongates during its poleward movement. Autosome movements, with only shortening fibres, seem to utilize actin and myosin [Fabian and Forer, Protoplasma 231 (2007) 79-85]. To test whether mechanisms for sex-chromosome movements might be similar, even though sex chromosomes are attached to both poles during their movements, we treated living crane-fly cells with inhibitors of actin (cytochalasin D, latrunculin B, and swinholide A), myosin (BDM), and myosin phosphorylation (Y-27632). We added drugs individually after sex chromosomes started to move poleward and then washed out the drug after 10 minutes. All anti-actin drugs altered movements: both sex chromosomes slowed, or stopped, and sometimes only one sex chromosome was affected. The anti-myosin drugs also slowed or stopped sex-chromosome movements, though for Y-27632 the movements generally recovered before it was washed out. The sex chromosomes often resumed movement at normal speed after the drugs were washed out. Overall, these results suggest that actin and myosin are involved in sex-chromosome anaphase movements in crane-fly spermatocytes. A next step is to study spindle fibres of sex chromosomes after immunostaining, to look for potential differences between shortening and elongating fibres.

369/B313

The Role of Arginylation for Actin Dynamics in Dictyostelium discoideum.

P. Batsios, M. Schleicher, A. Mueller-Taubenberger; Institute for Cell Biology, Ludwig Maximilians University, Munich, Germany

The social amoeba Dictyostelium discoideum is an excellent model organism to study cytoskeletal dynamics. The D. discoideum actinome comprises 41 actins and actin-related proteins, and 17 distinct actin genes code for identical proteins (Joseph et al., PLoS ONE 3(7), e2654, 2008). Fast actin rearrangements are regulated by actin binding proteins but little is known about the role of posttranslational actin modifications. Arginylation is a posttranslational modification that is only poorly understood at its molecular level. Arginylation of beta-actin was described to regulate actin filament properties, beta-actin localization, and lamella formation in motile cells (Karakozova et al., Science 313, 192, 2006). The highly conserved Arg-tRNA protein transferase (Ate1) is known to mediate arginylation. In order to test whether arginylation plays a role for actin-dependent processes in D. discoideum we knocked out the gene encoding Ate1 and investigated the phenotype of ate1-null cells. Similar to mouse fibroblasts deficient for Ate1 the Dictyostelium ate1-null cells are smaller in size as compared to wild type. Furthermore Dictyostelium ate1-null cells are much less adhesive. Random motility and chemotaxis were not significantly altered. Visualization of the actin cytoskeleton by live cell microscopy using the F-actin marker GFP-LimDelta indicated significant changes in comparison to wild type cells. Filament organization in the cortex was apparently unchanged, but the mutant lacked the actin spots at the substrate-attached surface. These actin spots are usually involved in clathrin-mediated endocytosis and cell adhesion. We are currently testing whether actin is in fact modified in D. discoideum and whether the differences observed in actin dynamics are directly or indirectly linked to arginylation.

370/B314

Role of Intermonomer Ionic Bridges in the Stabilization of the Actin Filament.

E. Stokasimov, M. McKane, P. Rubenstein; Biochemistry, University of Iowa, Iowa City, IA

Double-stranded filament formation by actin is required for most of actin's physiological functions. However, the intermonomer interactions resulting in stabilization of F-actin have not been elucidated because of a lack of an F-actin crystal structure. The Holmes muscle actin model suggests that an ionic interaction between R39 of one monomer and E167 of an adjacent monomer in the same strand contributes to this stabilization. Yeast actin has an A167 instead. F-actin molecular dynamics modeling predicts another interaction between R39 of one monomer and D275 of an opposing strand monomer. In Toxoplasma gondii actin, which forms short stubby filaments, the D275 equivalent is replaced by R causing a potential filament-destabilizing charge-charge repulsion. We made yeast actin A167E as a potential stabilizer and A167R as a potential disruptor. We also made D275R and tested the ability of mutant actin functions in yeast and in vitro. All mutations caused abnormal growth and mitochondrial malfunction. A167E and D275R actins polymerize normally and form relatively normal appearing filaments. A167R nucleates filaments more slowly and forms filament bundles. Stoichiometric amounts of yeast cofilin have little effect on WT and A167E filaments. However, D275R and A167R depolymerization is profound with cofilin. We were not successful in creating 275R/39D(E) double mutants in yeast actin. However, we were able to generate A167R/R39D mutant actin in yeast cells that also contained a specific mutation in the profilin gene. A167R/R39D mutant actin showed polymerization kinetics similar to that of WT actin, and EM showed normal appearing filaments and an absence of the bundles observed with A167R actin alone which could be decorated with cofilin. These results with A167R strongly support the formation of an R39-A167E ionic interaction. Our results further suggest that while disruption of the interaction between R39 and D275 is not sufficient to cause fragmentation, it changes filament stability thereby disposing it for enhanced cofilin depolymerizing effects. Collectively, these results demonstrate in vivo and in vitro the importance of potential R39 ionic interactions.

371/B315

Biochemical Effects of Deafness-causing γ-Actin Mutations on Actin Functions.

S. E. Bergeron[1], S. Korrapati[2], M. Zhu[2], P. Rubenstein[1]; [1]Biochemistry, University of Iowa, Iowa City, IA, [2]Microbiology and Molecular Genetics, Michigan State University, East Lansing, MI

Inner ear hair cells, which play a central role in sound detection, contain β and γ nonmuscle isoactins in a ratio of about 1:2, opposite of that found in most other cells. Eight point mutations in mammalian γ-nonmuscle actin cause autosomal dominant deafness. Although the disease mechanism is unknown, our previous work suggests these mutations do not cause overt defects in polymerization, many of the mutations lie in regions that may affect the binding of actin filament regulatory proteins. To address this hypothesis, we are utilizing a baculovirus system to express human actins containing these mutations for biochemical characterization. However, in order to establish a baseline for our comparison, we have initially focused on the comparison of the two normal nonmuscle actin isoforms themselves, since so little is known about their behavior. These two isoactins differ at only 4 residues out of 375. The N-terminal D-D-D- of β actin is replaced by E-E-E- in γ-actin, and V10 in β-actin is converted to I in γ-actin. Based on these differences, one would expect little if any difference in their properties. However, in motile cells, β-actin tends to be concentrated in very dynamic areas at or near the leading edge of the cell, whereas γ-actin is found predominantly in stress fibers. Our initial results show that their thermal stabilities and critical concentrations are approximately the same. Surprisingly, our initial results, using two different actin preparations, indicate that β-isoactin polymerizes very rapidly, with rates similar to yeast actin. Conversely, γ-isoactin polymerizes even more slowly than muscle actin, which correlates with their use in the cell. Preliminary EM results also show that the γ-isoactin forms straighter and longer filaments than does β-actin, again consistent with their cellular roles. These results indicate that, although the N-terminal actin acidic residues are in a seemingly unstructured part of the molecule, away from monomer-monomer interfaces, within the filament, they may allosterically regulate these interfaces leading to the polymerization and filament structural differences we observe. How this occurs will be the focus of future studies.

372/B316

Characterization of the Human Cardiac Actin Variant M305L.

R. W. Demers, M. M. Mundia, J. Dawson; Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada

Mutations in the human cardiac actin gene ACTC have been implicated in the development of heart disease. Seven of these mutations are associated with hypertrophic cardiomyopathy (HCM: E99K, P164A, Y166C, A230V, A295S, M305L and A331P), and two are associated with dilated cardiomyopathy (DCM: R312H and E361G). We want to answer the question: What are the molecular defects associated with ACTC mutations that contribute to the development of heart disease? Defects may occur in the generation of force, the transmission of force, or the stability of the actin protein itself. The M305L mutation is located at the surface of action in the proximity of the putative myosin-binding site and may impair force generation. We have expressed and purified the HCM ACTC variant M305L with the baculovirus system. Using circular dichroism we show that the variant protein possesses a similar melting temperature (55.5°C) to recombinant wild-type ACTC protein (56.8°C). After 5 days, the M305L protein exhibits little change in its ability to inhibit DNase-I activity, relative to wild-type recombinant ACTC. Polymerization ability of the M305L variant was also assayed using pyrene fluorescence and dynamic light scattering. Critical concentration (Cc) for polymerization of M305L (2.66 μM) was found to be comparable to that of wild-type recombinant ACTC (2.16 μM). Taken together, these data suggest that the M305L protein stability and defects in actin intrinsic properties are not contributing to the development of HCM. We will therefore study the protein-protein interactions involved in force generation and transmission employing in vitro motility and actin-activated myosin ATPase activity assays.

373/B317

Polymerization Deficient PBM-crosslinked F-actin Protomers Show Conformational Transitions from Low to Moderate Ionic Conditions.

A. Perieteanu, J. Dawson; University of Guelph, Guelph, ON, Canada

Filamentous actin (F-actin) plays key roles in many physiological processes including organelle trafficking and muscular contraction. A high resolution structure of F-actin would provide key insights into filament nucleation, growth, dynamics, and interactions between F-actin and the myriad of F-actin binding proteins (ABPs). We have constructed and purified large quantities of crystallization grade polymerization deficient PBM-crosslinked actin dimers and trimers. Polymerization-competent actin dimers were shown to act as actin filament nucleators while polymerization-deficient dimers and trimers failed to do so. The inhibited actin dimers do retain their ability to bind ABPs such as gelsolin, gelsolin segment 1, DNase I, and myosin. DNase I assays yielded IC50 values of 10.6 ± 0.9 nM, 9.4 ± 1.4 nM and 22.2 ± 3.0 nM, for inhibited monomers, dimers and trimers under low ionic strength conditions and 21.1 ± 0.8 nM and 35.1 ± 3.0 nM for dimers and trimers under moderate ionic strength conditions. These data imply that moderate ionic strength conditions induce a conformational change from a loosely packed actin dimer or trimer under low ionic strength to a tightly packed actin dimer or trimer possessing fewer DNase I binding sites. To validate this model, thermal unfolding, nucleotide exchange and protease digestion studies are currently being undertaken.

374/B318

Carbon Nanotubes Alter Actin Organization and Reduce Cell Proliferation.

K. N. Dahl[1,2], M. F. Islam[2,3]; [1]Department of Biomedical Engineering, Carnegie Mellon University, Pittsburgh, PA, [2]Department of Chemical Engineering, Carnegie Mellon University, Pittsburgh, PA, [3]Materials Science and Engineering, Carnegie Mellon University, Pittsburgh, PA

The ability of actin to form filaments and reorganize into higher-order network structures is vital for cells to perform essential functions such as force generation, motility, division and maintaining cell shape. Thus far, natural chemical approaches have been utilized exclusively to alter actin polymerization and filament organization. We report observations of dramatic actin bundle formation in cells and in vitro induced by introduction of purified and isolated single-wall carbon nanotubes (SWCNTs). These specially prepared SWCNT treated cells do not kill cells, but cell proliferation is significantly reduced. Treated cells also show an increase in division defects. Images obtained using laser scanning confocal microscope show that actin is redistributed from basal focal adhesions in control cells to heterogeneous structures throughout the SWCNT-treated cell. Experiments with purified actin show SWCNT-induced actin bundling by both low-speed centrifugation and confocal imaging. The anisotropic nature of SWCNT bundles versus α-actinin induced isotropic networks suggests that SWCNTs promote lateral actin association by a physical interaction due to their size anisotropy and stiffness. Actin bundling in the presence of SWCNTs coupled with the potential for SWCNT modification and targeting has unlimited potential for cellular manipulation in cell biology research and medicine.

Mitosis and Meiosis I (375-396)

375/B322

Chromosomal Scale Alterations in Pluripotent and Differentiated Mammalian Cells.

J. Schaub[1], T. Stearns[2,1]; [1]Cancer Biology Program, Stanford University, Stanford, CA, [2]Dept. of Biology, Stanford University, Stanford, CA

Proper replication, repair, and segregation of chromosomes, termed genome stability, are essential to cell and organism function. Failure of any of the mechanisms of genome stability can result in defects ranging from small mutations, such as single base changes or small insertions/deletions, to large chromosome abnormalities, such as translocations and chromosome gain or loss (aneuploidy). Genome instability is associated with cancer, and tumor cells often have grossly abnormal genotypes. One result of genome instability is loss of heterozygosity, in which an existing heterozygosity in a diploid individual is reduced to homozygosity or hemizygosity through mitotic recombination, chromosome loss, deletions/insertions, or point mutations. Loss of heterozygosity is a particularly common form of genome instability in which loss of one allele of a gene can reveal recessive phenotypes in a heterozygous individual. This loss of heterozygosity plays a large part in many cancers. Although the proteins, pathways and processes related to genome stability have been well studied in simple model systems, much less is known in mammals. We are developing an in vivo assay system that allows for the detection of loss of heterozygosity in individual cells, and differentiation of chromosomal scale alterations (CSA) from point mutations. In this assay system the gene for green fluorescent protein (GFP) at one locus is under the negative control of the tetracycline (Tet) repressor, expressed from a separate locus. If the Tet repressor is lost by a loss-of-heterozygosity event (mutation, mitotic recombination, or chromosome loss), GFP is expressed. A second fluorescent marker is placed at the Tet repressor locus to distinguish between intragenic events, affecting only the Tet repressor gene, and CSAs. We are establishing this assay in mouse embryonic stem cells to enable examination of CSAs in differentiated and self-renewing cells and the comparison of levels among different cell types, with the ultimate goal of using this assay system to determine the extent, location, and timing of genomic instability in wild-type and cancer model mice.

377/B324

Developing the Planarian S. mediterranea as a Model Organism for Meiosis.

J. Chretien, A. F. Dernburg; Jacqueline Chretien, UC Berkeley, Berkeley, CA

The planarian Schmidtea mediterranea is an emerging model organism, especially favored for the study of regeneration and cell fate specification. S. mediterranea is a member of an understudied phylogenetic group, the lophotrochozoa, and shares a significant number of genes with humans that have been lost in C. elegans or D. melanogaster (Newmark 2002). Meiosis in planarians is particularly fascinating; these organisms can stably propagate as diploids or triploids, and appear to have extremely flexible mechanisms for chromosome pairing and segregation in meiosis. Currently, we are interested in understanding how telomere clustering in meiosis (the "bouquet") is mediated by cytoskeletal elements, telomere associated proteins and other meiotic proteins, and what the consequences of disrupting the bouquet are on subsequent events in meiosis. I am using cytological methods in diploid S.mediterranea testes to investigate the basis for bouquet formation and later events in spermatogenesis. This study takes advantage of recently developed resources, such as RNAi knockdowns and a sequenced genome, and also demonstrates a new cytological technique for S. mediterranea.

378/B325

Investigating the Functions of Meiotic Gene Homologs in Giardia intestinalis.

M. L. Carpenter[1], M. Poxleitner[1], J. J. Mancuso[1], S. C. Dawson[2], Z. Cande[1]; [1]Molecular and Cell Biology, UC Berkeley, Berkeley, CA, [2]Microbiology, UC Davis, Davis, CA

The divergent diplomonad parasite *Giardia intestinalis* contains two functionally equivalent nuclei that are inherited independently during mitosis. Although presumed to be asexual, *Giardia* has low levels of allelic heterozygosity, indicating that the two nuclear genomes may exchange genetic material at some point in the life cycle. Using fluorescence in situ hybridization (FISH) and electron microscopy, we have found that the nuclei can fuse and exchange episomes during encystation, a parasexual process we have termed "diplomixis." Furthermore, GFP-fusions of giardial homologs of meiosis-specific genes localize to the nuclei of cysts, but not the vegetative trophozoite, suggesting that homologous recombination may occur during diplomixis. To test this hypothesis, we have begun integrating chromosomal markers that can be tracked during encystation. Additionally, we have developed morpholinos as a tool to knock down genes in *Giardia*, achieving up to 80% knockdown of GFP in 12 hours that lasts at least 72 hours. We are now applying this technology to the meiosis-specific gene homologs to determine their functions. The results of these studies will be important for understanding how *Giardia* maintains two equivalent nuclei, as well as for understanding the evolution of DNA damage repair and meiosis.

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Regulation of Sister Chromatid Cohesion during C. elegans Meiosis.

M. Schvarzstein[1], E. Martinez-Perez[1,2], A. Villeneuve[1]; [1]Developmental Biology, Stanford University, Stanford, CA, [2]Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield, United Kingdom

Temporal and spatial regulation of sister chromatid cohesion loss during the meiotic divisions is essential to allow sequential separation of homologous chromosomes during anaphase I, followed by separation of chromatids in anaphase II. We are investigating how this two-step loss of sister chromatid cohesion is accomplished during meiosis in *C. elegans*, where a single crossover divides the homolog pair (bivalent) into two distinct subdomains: a short axis, where sister chromatid cohesion will be released at anaphase I, and a long axis where sister chromatid cohesion will be retained until anaphase II. Whereas most chromosome axis components localize to both the long and short axes of the bivalent, we observed that HTP-1/2 proteins are restricted to the long axis, where sister chromatid cohesion is retained during anaphase I. Further, HTP-1/2 are abruptly removed from the chromosomes at the onset of anaphase I, right after the sister chromatid cohesion loss from the short axis that allows homolog separation. Therefore, the spatial-temporal localization of HTP-1/2 is suggestive of a role protecting sister

chromatid cohesion along the long axis of the bivalent until the second meiotic division. Moreover, *htp-1 htp-2* mutants exhibit both premature removal of the cohesin component REC-8 from chromosomes and a premature separation of sister chromatids. These and other data indicate that HTP-1/2 regulates the two step-loss of cohesion during meiosis by maintaining localized chromatid cohesion between chromatids on the long axis of the bivalent until anaphase II. An additional mode of regulation of sister chromatid cohesion was suggested by our high-resolution image analysis of bivalents. We found that REC-8 is organized in a loop-like structure on each long arm of the bivalent. We understand this organization of REC-8 on each homolog to represent two sister chromatid cores that are separated along most of their lengths, but remain tightly associated at the bivalent ends pointing towards the spindle poles at metaphase I. We interpret that this bivalent organization during prophase I may facilitate both alignment at the metaphase II plate and rapid release of sister chromatid cohesion at anaphase II.

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Keeping Sisters Linked: Axial Element Components Promote Sister Kinetochore Co-orientation in C. elegans Meiosis.

A. Severson, V. van Zuylen, L. Ling, B. Meyer; Molecular and Cell Biology and HHMI, University of California -- Berkeley, Berkeley, CA

In most organisms, homologous chromosomes separate in meiosis I, while the two sister chromatids per homolog remain linked until meiosis II. Keeping sisters together requires that cohesion between sisters be protected during meiosis I and the two kinetochores of each homolog capture MTs from a single spindle pole (co-orient). We show that in *C. elegans*, the maintenance of sister chromatid cohesion and the co-orientation of sister kinetochores require not only the meiotic cohesin subunit REC-8, but also, unexpectedly, HTP-1, -2, and -3, three orthologs of the yeast axial element component Hop1p. In animals deficient for REC-8, homologs remain separate. Sister kinetochore co-orientation fails in anaphase I, and the two sister chromatids per homolog segregate toward opposite poles (equational division). Zygotes generally inherit one chromatid from each homolog. Chromosome segregation then fails in meiosis II, and the progeny of rec-8 mothers fertilized by wild-type males survive as triploids. Homologs also remain apart in *spo-11* mutants, which fail to initiate crossover recombination, but sister chromatids do not separate equationally in meiosis I. Instead, homologs segregate randomly and most embryos die with aneuploid genomes. Remarkably, disrupting sister kinetochore co-orientation by disrupting REC-8 restores viability to *spo-11* mutants. Hence, we screened for suppressors of *spo-11* lethality to identify additional factors required for kinetochore co-orientation. The first suppressor identified (*y428*) harbors a premature stop codon in *htp-3*. Depletion of HTP-3 by RNAi similarly suppresses *spo-11* and results in an equational division at meiosis I. Surprisingly, the cohesin subunit SMC-1 was undetectable on chromosomes throughout meiosis I in *htp-3(y428)* mutants and *htp-3(RNAi)* animals, suggesting an unexpected role for axial elements in the loading or retention of cohesin on meiotic chromosomes. Simultaneous depletion of HTP-1/HTP-2 also suppresses *spo-11* and permits equational separation of sisters during anaphase I. Our data demonstrate a novel role for axial elements in kinetochore co-orientation, involving either recruitment or stabilization of cohesin, or a second, unknown mechanism.

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Regulation of Homolog Pairing and Synaptonemal Complex Formation in C. elegans Meiosis.

M. Zastrow[1], M. Hayashi[1], N. Miley[1], A. J. MacQueen[2], A. Villeneuve[1]; [1]Developmental Biology, Stanford University School of Medicine, Stanford, CA, [2]Molecular, Cellular, and Developmental Biology Department, Yale University, New Haven, CT

Meiosis, the specialized cell division program that generates haploid gametes from diploid germ cells, is essential for sexual reproduction. Meiosis begins with an extended prophase during which homologous chromosomes undergo a sequence of events that prepare them to segregate to opposite poles at the meiosis I division. One such event is a dramatic clustering of chromosomes that is predicted to promote two genetically separable yet coordinated events: 1) recognition and alignment of homologous chromosomes and 2) stabilization of this alignment by the proteinaceous synaptonemal complex (SC). The SC is comprised of two longitudinal chromosomal axes, which are assembled along the length of each chromosome upon entry into meiotic prophase, and a central region that connects the axes of paired and aligned chromosomes. We are investigating mechanisms that coordinate homolog pairing and SC assembly, focusing on the role of HAL-2, a novel component of the meiotic machinery. hal-2 mutants fail to cluster chromosomes at the onset of prophase and exhibit severely reduced levels of homolog pairing. In addition to defective pairing, hal-2 mutants incorrectly load the central region protein SYP-1 on unpaired chromosomes. This property distinguishes hal-2 mutants from chk-2 (previously known as hal-1) mutants, in which chromosome clustering and pairing are defective but SYP-1 is prevented for loading on unpaired homologs, and htp-1 (a paralog of the axial element HIM-3) mutants, which load SYP-1 to form improper SC between non-homologous chromosomes. Further, hal-2;chk-2 and hal-2;htp-1 double mutants resemble hal-2 single mutants with respect to loading of SYP-1 on unpaired chromosome axes. Moreover, hal-2;syp-2 double mutants have an increase in chromosome pairing compared to hal-2 single mutants indicating that inappropriate loading of SYP proteins is at least partially responsible for inhibiting chromosome pairing. Our results imply a role for HAL-2 in a mechanism that restricts the loading of SC central region proteins only upon properly paired homologous chromosomes.

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Matefin/SUN-1 Is Required for Homologous Chromosome Pairing in C. elegans Meiosis.

A. Penkner[1], A. Fridkin[2], J. Gloggnitzer[1], A. Baudrimont[1], E. Csaszar[3], P. Pasierbek[4], Y. Gruenbaum[2], V. Jantsch[1]; [1]Dept. of Chromosome Biology, University of Vienna; Max F Perutz Labs, Vienna, Austria, [2]Department of Genetics, Hebrew University of Jerusalem, Jerusalem, Israel, [3]Mass Spectrometry Facility, University of vienna; Max F Perutz Labs, Vienna, Austria, [4]Dept. of Biopotics, Research Institute of Molecular Pathology, Vienna, Austria

Faithful chromosome partitioning during meiosis relies on the correct recognition and stable pairing of parental homologous chromosomes during meiotic prophase I. The identification of a highly specific mutation in the germ line-restricted nuclear envelope protein matefin/SUN-1 allowed us to uncover the active contribution of the nuclear envelope to the process of homologous chromosome alignment and pairing in C. elegans meiosis [1]. The inner nuclear envelope protein matefin/SUN-1 contains a conserved SUN domain that presumably interacts with the outer nuclear envelope protein ZYG-12, thereby establishing a proteinaceous bridge across the nuclear envelope that connects components in the nucleus to cytoplasmatic structures. Time-lapse analysis of matefin/SUN-1::GFP transgenes demonstrated that the mutated

SUN-domain abrogates movement of matefin/SUN-1 patches, most likely representing an equivalent to chromosomal attachment plaques. This nuclear/cytoplasmatic link is therefore facilitating chromosome movement and prevents non-homologous synapsis. Remarkably in leptotene/zygotene, right at the stage when meiotic chromatin reorganizes and homologous chromosome contacts are initiated, specific changes in the characteristics of matefin/SUN-1 can be observed. Mass spectrometry analysis revealed that the nucleoplasmic N-terminal domain of matefin/SUN-1 becomes phosphorylated at multiple serines. We will report on the mutational analysis of the potential phosphorylation sites by studying their impact on homologous chromosome pairing and other meiotic events. Our data so far suggest that the properties of the nuclear envelope are changed specifically during leptotene/zygotene, the time window when homologs get sorted and matefin/SUN-1 patches are formed and that those changes influence the movement of chromosomes. [1] Penkner et al. (2007) Dev. Cell 12, 873-85.

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The Crossover Machinery Inhibits Aberrant Synapsis in C. elegans Meiosis.

S. E. Rodenbusch[1], N. Bhalla[2], A. F. Dernburg[1]; [1]Molecular and Cell Biology, The University of California, Berkeley, CA, [2]University of California, Santa Cruz, CA

Accurate chromosome segregation in meiosis requires the stabilized pairing of homologs. In most organisms, pairing is stabilized by the polymerization of the Synaptonemal Complex (SC) and by the formation of crossovers. During early meiosis, double strand breaks (DSBs) initiate recombination within the context of the SC. Each DSB is resolved either as a “simple” gene conversion event or as a reciprocal exchange, known as a crossover. The relationship between SC polymerization, also known as synapsis, and crossover formation is intimate but varies among organisms. In some organisms, such as S. cerevisiae and mice, mutations that disrupt recombination also disrupt synapsis, and defects in synapsis reduce the number of crossovers. However, in both Drosophila and C. elegans, recombination deficient mutants still successfully pair and synapse. Although these processes can apparently be uncoupled in C. elegans, we wanted to re-examine their relationship. To this end, we characterized recombination mutants in a sensitized background in which pairing and synapsis of a single chromosome is disrupted. In this background, we find that spo-11 mutants, which fail to catalyze DSBs, cause a surprising and dramatic increase in synapsis of the affected chromosome. Close examination of these synapsed chromosomes by fluorescence in situ hybridization (FISH) reveals that many of them have synapsed non-homologously or by folding over on themselves. We find a similar phenotype with mutations in several other genes that are required for the formation and resolution of crossovers: rad-51, zhp-3, and msh-5. Our findings indicate that the crossover machinery in C. elegans may not be necessary to promote the correct loading of SC, but instead to inhibit the aberrant loading of SC between non-homologous chromosome regions.

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Analysis of an Attached X Chromosome Reveals Links between Homolog Pairing and Synapsis Initiation in C. elegans Meiosis.

R. Kasad[1,2], A. F. Dernburg[1,2]; [1]Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, [2]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

During early meiotic prophase, the environment of the nucleus is dynamic, as a given chromosome will undergo a series of encounters with homologous and nonhomologous chromosomes. A proteinaceous structure called the synaptonemal complex forms in between chromosomes to stabilize specifically inter-homolog interactions. Homolog pairing and synapsis are both essential for proper segregation of chromosomes. A fundamental problem is how each chromosome is able to discriminate and accurately initiate synapsis only with its appropriate partner. In *C. elegans*, *cis*-acting chromosomal loci and a family of zinc-finger proteins, pairing center (PC) proteins that bind to these loci, are essential for establishing homolog pairing. Genetic lesions that disrupt PC proteins result in pairing defects and missegregation of chromosomes. These proteins clearly play a role in stabilizing homolog interactions, but their role in synapsis initiation remains unknown. In addition to chromosomal elements, cytoskeletal components have also been implicated in pairing and synapsis. Microtubules and motor proteins, via connections to PCs through specific nuclear envelope components, seem to provide a mechanical force to facilitate pairing and synapsis. Through analysis of a strain carrying an artificially paired set of *X* chromosomes, we bypass the need of the PC proteins' function in pairing and are able to examine their role in synapsis initiation. Our results indicate that PC proteins are required for synapsis initiation. We also note that in our modified system where pairing is artificially achieved, nuclear envelope components do not seem to be as critical for promoting synapsis. We also find evidence that PC proteins play a role in inhibiting synapsis initiation between nonhomologs.

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Identifying Novel Nuclear Envelope-associated Components Involved in Pairing and Synapsis of Meiotic Chromosomes in C. elegans.

R. Rillo, A. F. Dernburg; Molecular and Cellular Biology, UC Berkeley, Berkeley, CA

We are interested in understanding the mechanism of chromosome pairing and synapsis of meiotic chromosomes. Previous studies in the Dernburg lab have revealed the roles of Pairing Centers (PCs), cis-acting elements on each chromosome. A family of zinc-finger proteins binds to these PCs and mediate pairing and synapsis. This family of proteins, known has HIM-8, ZIM-1,-2,-3, mediates association of specific chromosomes to the nuclear envelope during meiotic prophase. Recent, unpublished work from the lab has identified specific nuclear envelope proteins that interact with the PCs. These include SUN-1 and ZYG-12, transmembrane proteins that localize to the inner and outer nuclear membrane, respectively. In many organisms, including *C. elegans*, chromosome clustering, also known as the ‘bouquet stage’, plays an important role in the process of pairing and synapsis. A mutation in a nuclear envelope component, like SUN-1 and ZYG-12, leads to a decrease in associations between homologous chromosomes, defects in synapsis, and an increase in inappropriate interactions between nonhomologous chromosomes. Although we do not yet understand how exactly these nuclear envelope components are coordinating pairing and synapsis, my goal is to identify novel components that may contribute to these processes. I have used a meiosis-specific allele of *sun-1*, whose phenotype leads to nonhomologous synapsis and 100% inviable progeny, in a suppressor screen to identify genetic interactors. As a complementary approach, I am working to identify components that physically interact with SUN-1 by co-immunoprecipitation. Identifying novel nuclear envelope components may lead to our better understanding of the bouquet stage in *C. elegans*, and how this leads to appropriate pairing and synapsis of meiotic chromosomes.

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Regulation of Nuclear Reorganization in C. elegans Meiotic Prophase.

N. Harper, A. F. Dernburg; Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

To ensure their proper segregation at the first meiotic division, homologous chromosomes must accurately pair, synapse and recombine during meiotic prophase. Although the regulation of homolog pairing remains mysterious, evidence from many organisms indicates a role for chromosome reorganization in the process. In the *C. elegans* gonad, homolog pairing occurs in the transition zone when chromosomes are polarized to one side of the nucleus. Recent work has shown that chromosomes are tethered to the nuclear envelope at protein patches that aggregate in transition zone nuclei. These transition zone patches span the nuclear envelope, connecting chromosomes to the cytoplasmic microtubule network and promoting homolog pairing and synapsis. Chromosomes are tethered to the nuclear envelope through the HIM-8 and ZIM family of proteins that bind specifically to Pairing Centers of chromosomes and localize to the transition zone patches. The inner nuclear envelope component SUN-1 and its binding partner in the outer nuclear envelope, ZYG-12 link nuclear and cytoplasmic patch components. Members of the dynein motor complex bind to ZYG-12 allowing microtubule forces to act through the patches to influence nuclear organization and chromosome dynamics. Interestingly, the nuclear envelope proteins SUN-1 and ZYG-12 must relocate from anchoring premeiotic centrosomes at the nuclear envelope to form these transition zone patches in meiosis. Although it remains unclear how this gross reorganization of patch components and chromosomes occurs, a likely regulator of these processes is the checkpoint protein kinase, CHK-2. CHK-2 is required for transition zone patch formation, chromosome polarization, homolog pairing, synapsis and recombination; however, the mechanism of CHK-2 function remains unknown. Here, we report that CHK-2 localizes to premeiotic centrosomes and relocates to transition zone patches in meiotic prophase. Based on this localization pattern, we are investigating the hypothesis that CHK-2 may directly regulate the reorganization of nuclear envelope components to mediate meiotic chromosome dynamics.

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Characterization of Mechanisms Involved in Establishing Nuclear Reorganization during Meiosis.

C. Kotwaliwale, A. F. Dernburg; UC Berkeley, Berkeley, CA

Chromosome missegregation results in genomic instability, which is implicated in tumorigenesis. In mitotically growing cells, sister chromatids associate with each other until anaphase onset. The association between sister chromatids is established concomitantly with DNA replication and tightly regulated to enable their segregation to opposite poles during anaphase. Meiosis poses an additional challenge that it requires interactions between homologous chromosomes, which must be established de novo during this process. Segregation of homologs to opposite poles during the first meiotic division requires that chromosomes first pair, or find and recognize their homologous partners, among a pool of other chromosomes. At the onset of meiosis, homologs occupy distinct domains within the nucleus. The mechanism by which chromosomes contact and identify their partners has remained elusive despite extensive effort. In C. elegans, a family of zinc finger proteins is essential for homolog pairing. Members of this family associate with cis-acting regions called "pairing centers" (PCs) located near one end of each chromosome. Prior to pairing, chromosomes associate with the nuclear envelope via the PCs and undergo transient spatial reorganization, resulting in the clustering of chromosomes towards one side of the nucleus. One possibility is that chromosome clustering promotes the "search and capture" of homologs, resulting in pairing. Consistent with this, mutants that are defective in chromosome clustering are also defective in homolog pairing. A key goal is therefore to determine the mechanism by which chromosomes associate with the nuclear envelope and cytoskeleton. To this end, we conducted a genetic screen using a fluorescent nuclear envelope marker to identify components involved in chromosome dynamics during early meiosis. We present here an initial characterization of mutants isolated in this screen and discuss the implications of our findings for our current understanding of homolog pairing.

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Meiotic Spindle Assembly and Function in C. elegans.

M. H. Price, V. Davis Haug, B. Bowerman; Institute of Molecular Biology, University of Oregon, Eugene, OR

Female meiotic cell divisions generally use acentrosomal microtubule spindles, but the mechanisms that govern their assembly remain largely unknown. Female meiotic cell divisions in *C.elegans* provide an excellent model to understand meiotic spindle function, with powerful genetics and GFP fusions to facilitate live cell imaging of meiosis I and II (MI & MII) in utero. Although wild-type spindle dynamics in oocytes have been described in detail (Yang et al. 2003. Dev Biol.), little is known about the genetic pathways that orchestrate meiotic spindle assembly and function. To better understand female meiotic cell divisions, we performed forward genetic screens and isolated seven temperature-sensitive, embryonic-lethal, recessive mutants in which we detect multiple maternal pronuclei in one-cell stage embryos. Two, *or642* and *or646*, are alleles of *mei-1*, which encodes a katanin subunit; one, *or447*, is an allele of the kinesin gene *klp-18*. Another, *or645*, is an allele of *aspm-1*, the *C.elegans* ortholog of human ASPM (abnormal spindles and primary microcephaly); little is known about its roles during female meiotic divisions in any species. Three mutations (*or600*, *or627*, *or643*) map to small intervals. Because *or627* and *or643* do not map near genes required for meiotic divisions, we think these requirements were missed in genomewide RNAi screens. We are examining spindle assembly in these mutants using spinning disk microscopy with GFP::β-tubulin and mCherry::Histone. During MI in *or627* mutants, multiple unstable MTOCs form but never coalesce into a stable bipolar spindle. Rather, the MTOCs frequently converge to generate a monopolar spindle that extrudes all chromosomes into one polar body. We also are using recombineering to generate GFP fusions to 12 proteins known to be required for meiotic chromosome segregation. We will use these fusions to further dissect meiotic spindle assembly and function, using both RNAi and our mutants to deplete gene functions. One example, ASPM-1::GFP, is enriched at spindle poles during anaphase. Progress on our efforts to further dissect meiotic spindle assembly will be reported.

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Communication between Sex Chromosomes in Latrodectus mactans Male Meiosis Depends on Distance between Chromosomes.

R. Doan, L. V. Paliulis; Biology, Bucknell University, Lewisburg, PA

Lactrodectus mactans (black widow spiders) have a sex determination mechanism in which females are X1 X1 X2 X2, and males are X1 X2 O. In male meiosis, both the X1 and the X2 chromosomes move so they are immediately adjacent to the same pole of the cell before

anaphase I without ever aligning on the metaphase plate. This nonrandom nondisjunction of X1 and X2 can be explained because X1 and X2 are weakly connected to one another in meiosis I. This weak connection is easily disrupted using a micromanipulation needle. We find that, even after their weak connection is disrupted, X1 and X2 segregate together to the same pole when placed at the center of the spindle. However, we find that, when chromosomes are placed very far apart, they will associate with opposite spindle poles. When X1 and X2 were separated and induced to form connections with opposite poles, and one X was then detached from its newly associated pole and placed near the center of the spindle, that chromosome moved toward the other X chromosome on the spindle. When they are far apart, either they cannot communicate with one another, or they can communicate, but to no effect. If they can receive information from one another but do not respond, this is likely because chromosomes are stuck very close to their associated spindle pole and have no opportunity to interact with microtubules from the opposite pole. Therefore, the interaction and control of the sex chromosomes during meiosis I is dependent largely on location and distance of separation between X1 and X2.

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Cytoskeletal Forces Span the Nuclear Envelope to Coordinate Meiotic Chromosome Pairing and Synapsis.

A. Sato[1], C. M. Phillips[1,3], R. Rillo[1], R. Kasad[1], A. F. Dernburg[1,2]; [1]UC Berkeley, Berkeley, CA, [2]Lawrence Berkeley National Lab, Berkeley, CA, [3]Harvard Medical School, Boston, MA

Sexual reproduction requires the unique cell division called meiosis, in which a diploid cell undergoes a reductional division to produce haploid gametes. A hallmark of meiotic prophase is the formation of pair wise linkages between homologous chromosomes, which enables them to separate accurately from each other. In most organisms the pairing of homologous chromosomes is reinforced by synapsis, the polymerization of a protein scaffold called the synaptonemal complex (SC). The primary question addressed in this work is how pairing and synapsis are coordinated so that SC forms selectively between homologues. Specific chromosome sites known as Pairing Centers (PCs) are essential for homolog pairing and synapsis in C. elegans. Our laboratory has identified a family of four related proteins, HIM-8, ZIM-1, ZIM-2, and ZIM-3, that bind to the PCs on each chromosome and mediate homologous synapsis. Interestingly, foci of these proteins are associated with the nuclear envelope during early meiotic prophase and nucleate distinct patches of the nuclear envelope proteins, SUN-1 and ZYG-12. We provide evidence that a bridge comprised of SUN-1 and ZYG-12 connects meiotic chromosomes across the nuclear envelope to cytoplasmic microtubule motors. This connection coordinates proper pairing and synapsis between homologous chromosomes. Our analysis reveals the existence of a mechanism that “licenses” paired chromosomes to initiate synapsis, analogous to a checkpoint that acts during mitosis to coordinate chromosome attachment and spindle dynamics.

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The Spindle Checkpoint and Aneuploidy in Meiosis in Galleria mellonella Spermatocytes.

A. Asimoglou[1], S. Duponchel[1], S. Woods[2], M. Vega[2], L. V. Paliulis[1]; [1]Biology, Bucknell University, Lewisburg, PA, [2]Natural Sciences, Fayetteville State University, Fayetteville, NC

During cell division, it is essential that chromosomes are distributed flawlessly to each daughter cell. Failure to distribute chromosomes correctly results in aneuploidy (missing or extra chromosomes). Aneuploidy is prevented by the spindle checkpoint, which ensures that all chromosomes have attached to the spindle before anaphase. We study aneuploidy using Galleria mellonella (the greater wax moth) spermatocytes as a model system. Most moths undergo dichotomous spermatogenesis, producing apyrene and eupyrene sperm. Apyrene sperm are aneuploid and are produced following meiosis in which chromosomes fail to congress to the metaphase plate. Eupyrene sperm are haploid and are produced following normal meiosis. We hypothesize that apyrene spermatocytes have a defective spindle checkpoint, leading to congression failure and aneuploidy. To test this hypothesis, we have used micromanipulation and immunofluorescence to determine that eupyrene spermatocytes have a functioning spindle checkpoint. We have determined that eupyrene spermatocytes will delay anaphase until chromosomes that have been detached from the spindle realign on the metaphase plate. We have also shown that, in eupyrene spermatocytes, chromosomes with bipolar attachments to the spindle have low levels of fluorescence following staining with the 3F3/2 antibody, while unattached chromosomes have strong fluorescence. Apyrene spermatocytes are insensitive to micromanipulation and will progress into anaphase with any number of improperly attached chromosomes, thus suggesting a weak or nonexistent spindle checkpoint.

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Meiotic Chromosome Architecture and Homologous Synapsis Revealed by Ultrahigh Resolution Structured Illumination Microscopy.

C. Wang[1], P. Carlton[2], J. Sedat[2], Z. Cande[1]; [1]Molecular and Cell Biology, UC Berkeley, Berkeley, CA, [2]Department of Biochemistry and Biophysics, UCSF, San Francisco, CA

During leptotene of meiotic prophase I, each chromosome develops a linear proteinaceous structure called an axial element (AE). At leptotene-zygotene transition, telomere bouquet forms, which is accompanied by a transient remodeling of chromatin architecture. Meanwhile, double strand breaks occur to initiate homology search. In zygotene, the homologues synapse via the polymerization of a central element between the two homologous AEs, forming the synaptonemal complex (SC). During pachytene, synapsis and recombination are completed. These events are well known cytologically, and have been explored with light microscopy and EM. However, little is known about the kinetics of synapsis and changes in the organization of chromosome axes during synapsis. Structured illumination (SI) is a method of ultrahigh resolution light microscopy that overcomes the 250 nm limit of resolution of conventional light microscopy, and reaches at least 100 nm resolution in the x, y and z axes. We used SI to explore the organization of axial elements during synapsis and the pachytene chromosome architecture by monitoring the distribution of two axial element antibodies, AFD1 and HOP1 and several histone modification antibodies. The two lateral-element strands spacing 150 nm are clearly resolved and they twist around each other and have a left handed helical pitch mostly along whole chromosomes. About 76% of the unsynapsed regions in late-zygotene are associated with interlocks, implying that the resolution of interlocks between chromosomes may be a rate limiting step to complete synapsis. Many interlocks involving two unsynapsed non-homologous chromosomes are associated with non-homologous synapsis. In addition, those unsynapsed regions are very different in length, suggesting that the lateral element and chromosome itself are dynamic structures and that pre-synapsis adjustment in

length occurs during synapsis. We observed pachytene chromosomes and found the euchromatin stained by histone H3K4-dimethylated antibody forms thin and long fibers and heterochromatin stained by H3K9-demethylated antibody forms thick and compact fibers. The euchromatin fibers expand further than heterochromatin fibers.

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The Structural Maintenance of Chromosomes 5 and 6 Complex Confers Germline-specific Protection of Genomic DNA in C. elegans.

R. Chan[1,2], J. Bickel[1], J. Hayward[1], L. Chen[1]; [1]Human Genetics, University of Michigan, Ann Arbor, MI, [2]Internal Medicine-MMG Division, University of Michigan, Ann Arbor, MI

The Structural Maintenance of Chromosomes (SMC) proteins are highly abundant components of eukaryotic chromosomes. In general, SMC and associated proteins help to package and maintain the higher-order organization of chromosomes (1). This critical function ensures the accurate distribution of replicated chromosomes in mitosis and meiosis. There is emerging evidence for another critical role for SMC proteins in DNA damage response and repair (1, 2). The yeast and mammalian SMC-5/6 complexes, containing a heterodimer of SMC-5 and SMC-6 proteins and ancillary non-SMC subunits, have been implicated in DNA repair in the S and G2 phases of the cell cycle (2). We have recently characterized the nematode *Caenorhabditis elegans* SMC-5/6 complex in order to understand the biological functions of these conserved repair factors in metazoans. We generated specific antibodies to the *C. elegans* SMC-5 and SMC-6 proteins and examined their tissue-specific expression patterns during development and adulthood. We found the SMC-5/6 complexes are expressed in the germline of adult males and hermaphrodites, and they are highly enriched on meiotic chromosomes. The disruption of the SMC-5/6 complex severely reduced the production of fertilized egg, an indication of reduced sperm in hermaphrodites. Loss of the SMC-5/6 complex also induced germ cell apoptosis and excessive accumulation of the DNA repair factor RAD-51 during oogenesis. Together, these results provide the first functional demonstration in metazoans for the requirement of the SMC-5/6 complex in gametogenesis. Moreover, we discovered that the SMC-5/6 complex is highly expressed specifically in the germ cell lineage beginning at embryogenesis. The germ lineage accumulation and the conserved function of the SMC-5/6 complex in DNA repair suggest a possible role in maintaining germline genomic stability. We have examined a hypomorphic *smc-5* mutant worm strain and found additional evidence for increased germline mutation consistent with the hypothesis that the SMC-5/6 complex protects the germ cell lineage from accumulating genetic defects. 1. A. Losada, T. Hirano, Genes Dev. 19, 1269 (June 2005). 2. L. Strom, C. Sjogren, Curr. Opin. Cell Biol. 19, 344 (June 2007).

394/B341

Laser Ablation Reveals the Motile States of Kinetochores during Segregation of X-Y Sex Univalents in Crane Flies.

J. LaFountain[1], R. Oldenbourg[2]; [1]Biological Sciences, University at Buffalo, Buffalo, NY, [2]Marine Biological Laboratory, Woods Hole, MA

Our objective is to understand the mechanism by which univalent chromosomes -- arising from recombination failure or from precocious disjunction -- segregate during meiosis. Here we report results from a model system: the X-Y pair of sex chromosomes in crane-fly spermatocytes. X and Y disjoin at prometaphase onset and congress as univalents to the equator, both exhibiting amphitelic orientation (sister kinetochores connected to opposite poles). During anaphase A, X and Y lag at the equator. During anaphase B, they segregate to opposite poles, while retaining connections to both poles. Each univalent develops a leading and a trailing kinetochore (K-) fiber connected to opposite poles. The leading fibers shorten and the trailing fibers elongate, moving the two univalents to opposite poles. To ascertain the motile state of kinetochores on X and Y during anaphase A and B, we employed polarized light microscopy (LC-PolScope) in conjunction with a laser microbeam to ablate one kinetochore of a univalent and thus to detach it from one of its K-fibers. We then tracked subsequent motion mediated by the remaining intact kinetochore and its K-fiber using the LC-PolScope. During anaphase A, when both X and Y lag at the spindle equator, ablation of either one kinetochore of a univalent resulted in poleward (P) motion led by the intact kinetochore. However, during anaphase B, as X and Y segregated, ablation of the leading kinetochore stopped P-motion and the univalent remained stationary at the site of operation. On the other hand, ablation of the trailing kinetochore increased the speed of P-motion mediated by the leading kinetochore. We conclude from these results that the mechanism of X-Y segregation is achieved through the pulling action of leading fibers, resulting in P-motion of leading kinetochores. In that mechanism, trailing kinetochores and their K-fibers irreversibly shift to an essentially neutral (N) state, occurring during the transition from anaphase A to B, when the trailing kinetochore loses its potential for P-motion. Trailing fibers have the effect of resisting P motion of leading kinetochores and thus can account for the normal slow rate of movement of X and Y to their respective poles.

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Aurora-A Kinase Is an Interacting Partner for the NORE1A Growth Suppressor.

A. Moshnikova, A. Khokhlatchev; Pathology, University of Virginia, Charlottesville, VA

NORE1A (Novel Ras Effector 1 isoform A, also known as RASSF5) is an adaptor protein capable of binding activated Ras and Ras-related proteins in vitro and in vivo. NORE1A is expressed in most normal tissues but is lost in many tumors. Reconstitution of NORE1A expression induces growth arrest as well as cell death in a variety of cell lines. However, proteins that mediate NORE1A's effect on cell growth have not been identified and the molecular mechanism by which NORE1A induces growth suppression is unknown. We discovered that NORE1A is capable of interacting with the Aurora A, an evolutionary conserved serine-threonine protein kinase that is required for completion of cell division in eukaryotic cells. We propose that NORE1A associates with Aurora A kinase to induce growth inhibition and tumor suppression. This association could interfere with Aurora A function, leading to the inhibition of pre-cancerous cell growth or induction of apoptosis. We found that the interaction of NORE1A with Aurora A in transfected cells is moderately enhanced by activated (G12V) K-Ras. Studies with purified proteins demonstrated NORE1A:Aurora A interaction is direct and is mediated by two distinct NORE1A N-terminal domains. The Aurora A catalytic activity is not required for the NORE1A: Aurora A interactions. Both NORE1A and Aurora A are localized to centrosomes. Aurora A phosphorylates NORE1A at Threonine-277, a residue located in NORE1A's Ras-Associated domain. In transfected 293 cells, this phosphorylation regulated NORE1A interaction with activated Ras proteins. Co-expression of NORE1A with Aurora-A in 293 cells demonstrated that NORE1A is capable of inhibiting Aurora-A kinase activity. Thus, our data suggest Aurora A might be a relevant target for NORE1A-induced growth and tumor suppression.

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Phosphoproteomic Analysis of Mitotic Spindles Identifies Novel Integrin-linked Kinase Targets.

I. Dobreva[1], L. J. Foster[2], S. Dedhar[1,2]; [1]Cancer Genetics, BC Cancer Research Centre, Vancouver, BC, Canada, [2]Biochemistry and Molecular Biology, University of British Columbia, Vancouver, BC, Canada

Objective: Phosphorylation of intracellular proteins by protein kinases plays major role in the regulation of signal transduction pathways controlling vital biological processes. One protein kinase essential for the control of cell spreading, migration and survival is integrin-linked kinase (ILK). Some of its substrates include Akt/PKB and the phosphatase inhibitors PHI-1 and CPI-17. However, many of the ILK targets remain unidentified. We recently demonstrated that in addition to its role in cell adhesion and migration, ILK localizes to centrosomes/mitotic spindles and its inhibition or depletion disrupts the interaction between Aurora A kinase, TACC3 and ch-TOG, which is critical for mitotic spindle organization. This suggests that some mitotic spindle proteins may be ILK targets. Methods: We purified mitotic spindles from cells treated with the ILK-kinase inhibitor QLT-0267 or ILK-specific siRNA and applied TiO2-based phosphopeptide-enrichment in combination with liquid chromatography/mass spectrometry analysis to determine whether ILK inhibition or depletion leads to changes in the phosphoprotein composition of mitotic spindles. Results: LC-MS/MS analysis of mitotic spindles identified 140 phosphoproteins. Comparison between the phosphoprotein profiles of ILK-kinase inhibitor and ILK siRNA-treated spindles revealed that phosphorylation of 12 of the identified proteins was affected by both ILK inhibition and depletion. Some of the proteins that were affected include ensconsin, Tpx2, NUSAP1 and zyxin, previously suggested to play important roles in mitotic spindle integrity and cell division. Further analysis revealed novel phosphorylation sites in several of the newly identified proteins that have not been previously reported and that were affected by ILK inhibition or depletion. We are currently validating whether the newly discovered phosphoproteins are direct ILK targets and how changes in their phosphorylation status affect mitotic spindle integrity and cell division. Conclusions: By combining powerful technologies such as mass spectrometry with cellular fractionation and phosphopeptide enrichment methods we identified novel phosphoproteins in mitotic spindles, some of which may be direct ILK targets.

Cell Cycle Controls I (397-421)

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Coupling Cell Polarity with the Cell Cycle by a DYRK Kinase.

S. G. Martin, M. Berthelot-Grosjean; Center for Integrative Genomics, University of Lausanne, Lausanne, Switzerland

How does a cell sense its size and shape? And how does this affect cell cycle progression? We use the fission yeast, Schizosaccharomyces pombe, to unravel the mechanisms of cell proliferation and morphogenesis. As in all eukaryotes, entry into mitosis is controlled by the CDK1 kinase, whose activity is regulated by phosphorylation on tyrosine 15 (Y15). The wee1p kinase phosphorylates Y15 to inhibit CDK1, while removal of this inhibitory phosphate by the cdc25p phosphatase activates CDK1 to allow M phase entry. Wee1p and cdc25p are in turn controlled by upstream regulatory kinases. Rod-shaped fission yeast cells divide at constant size. The DYRK kinase pom1p is a key regulator of cell morphogenesis. Pom1p forms a gradient from cell tips and is essential for bipolar cell growth. It is also essential for medial division, acting as inhibitor of division site placement at cell ends. We have discovered that pom1p regulates entry into mitosis: pom1 deletion, or acute inhibition using an analogue-sensitive mutant bypass the G2 block conferred by cdc25ts mutant, while pom1 overexpression accentuates this block. This strongly suggests that pom1p functions as a dose-dependent G2-M inhibitor. Genetic epistasis experiments indicate that pom1p functions upstream of wee1p and inhibits cdr1p and cdr2p, two kinases of the SAD-1 family previously shown to inhibit wee1p. This inhibition is likely to be direct as pom1p phosphorylates cdr2p. Remarkably, cdr1p and cdr2p localize to a medial cortical region, which overlaps with the pom1p gradients from cell ends in small cells, but not long cells. Using protein fusions and morphogenesis mutants, we show that ectopic location of active pom1p away from cell ends confers a gain-of-function and imposes a G2 delay. These data suggest a model in which the extent of overlap between pom1p and cdr2p provides a measure of cell size to regulate M-phase entry. As DYRK and SAD-1 kinases are implicated in polarization and proliferation in multiple organisms, this suggests that this polarized network may have been conserved through evolution.

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Identification of fbxo25-associated Proteins Using a Tandem Affinity Tag for Two-Step Purification Approach.

F. R. Teixeira[1], C. Gartner[2], A. O. Manfiolli[1], S. Yokoo[1], A. Maragno[1], S. Gygi[2], M. Gomes[1]; [1]Biochemistry, University of Sao Paulo, Ribeirao Preto, Brazil, [2]Cell Biology, Harvard Medical School, Boston, MA

FBXO25 is one of 68 human F-box proteins that serve as specificity factors for a family of ubiquitin (Ub) ligase (E3-ligases) composed of SKP1, Rbx1, Cullin1 and F-box protein (SCF) that is involved in targeting proteins for destruction across the Ub-Proteasome System (UPS). FBXO25 proteins are concentrated in a dynamic subnuclear structure named FANDs (FBXO25-Associated Nuclear Domains) that overlapped with major nuclear sites of ubiquitination in cultured cells. We have used an improved tandem affinity purification method for purification of proteins interacting with FBXO25 in HEK293T cells. The modified TAP tag consists FLAG peptide and glutathione-S-transferase (GST) fused to the N-terminus of FBXO25. Combined with tandem mass spectrometry, the purification strategy allows us to identify around 100 proteins. Western blotting confirmed many of them including proteins of SCF complex (Skp1, Cullin1, FBXO25) and CAND1 (one regulator of this complex). Assays of in vitro ubiquitilation show that purified SCF complex containing FBXO25 was functionally active. In addition, a component of 19S proteasome, the Sug-1/Rpt6, identified in TAP, physically interacts with FBXO25. We demonstrate that FLAG and GST are two efficient tags for use in TAP purification system by to permit purified active complexes of proteins from the complex mixtures found in mammalian cell lysates. Moreover, a novel protein-protein interaction was identified between FBXO25 and Sug1. This method permitted to obtain interactome of FBXO25 and opened new perspectives to study of function this protein in cellular nuclei.

400/B347

ENOX, Cell Surface Copper-containing Proteins with Essential Roles in Cell Enlargement.

D. Morre[1], D. J. Morre[2]; [1]Foods and Nutrition, Purdue University, West Lafayette, IN, [2]Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN

Cells, once having divided, must enlarge to achieve some minimal size necessary to pass the G1 checkpoint that controls cell size in order to divide again. Little is known about how cells enlarge or the enzymes involved. Our laboratory has described a family of cell surface copper-containing proteins that have dual activities of hydroquinone oxidation and protein disulfide-thiol interchange that alternate. When blocked or inhibited, cell enlargement is also blocked or inhibited. When stimulated or overexpressed, cell enlargement is also accelerated. The activity directly involved in the enlargement process is that of the protein disulfide-thiol interchange. Antisera to the human constitutive ENOX protein of ENOX1 block both cell enlargement and protein disulfide-thiol interchange but have no effect on the rate of hydroquinone oxidase or oxidation of reduced pyridine nucleotide where NADH or NADPH serve as alternative substrates. Both activities are also sensitive to impermeant thiol reagents further implicating the interchange activity in the cell enlargement process. The constitutive ENOX1 is highly regulated and responsive to growth factor and hormonal controls. A cancer isoform, ENOX2, is constitutively activated and contributes to the cancer phenotype of unregulated cell growth. Published evidence suggests that the mechanism is not one of passive addition of new material to the growing cell surface but is an active process of force-extension in the ENOX proteins that acts in concert with an AAA-ATPase of the plasma membrane (Morré, et al., BioFactors 28:105-117, 2006).

401/B348

Autophagy Genes Mediate G0 Entry in Saccharomyces cerevisiae by the Selective Degradation of 14-3-3 Proteins.

M. Chen[1,2], A. Tassa[2], H. Cheong[3], C. Thomas[2], Y. Wei[1,2], A. Orvedahl[2], Q. Sun[2], C. De Virgilio[4], D. Klionsky[3], B. Levine[1,2]; [1]Howard Hughes Medical Institute, Dallas, TX, [2]Internal Medicine, University of Texas Southwestern Medical Center at Dallas, Dallas, TX, [3]Life Science Institute, University of Michigan, Ann Arbor, MI, [4]Medicine, University of Fribourg, Fribourg, Switzerland

Autophagy is an intracellular degradation system that delivers cytoplasmic constituents to the lysosome and also plays a wide variety of roles in physiology and pathophysiology. The mechanisms regulating starvation-induced cell cycle arrest in yeast and higher eukaryotes are incompletely understood. Here we show that several yeast autophagy genes, including ATG1, 5, 6, 7, 8 and 17, are required for S. cerevisiae to enter stationary phase (G0) in response to starvation. Yeast strains lacking autophagy genes display a defect in starvation-induced nuclear localization of the nutrient sensing kinase Rim15, which normally mediates G0 entry following its release from the 14-3-3 cytoplasmic anchor proteins, Bmh1 and Bmh2. Surprisingly, Bmh1/2 appear to be specifically targeted for autophagic degradation via a mechanism involving their binding to Atg6 during starvation. This targeting function of Atg6 is distinct from its function in autophagy, since an Atg6 variant harboring a mutation in the serine 195 phosphorylation site, while being fully functional for general autophagy, is defective for starvation-induced binding and degradation of 14-3-3 proteins, Rim15 nuclear localization, and proper G0 entry. Thus, Atg6 specifically targets 14-3-3 proteins for autophagic degradation during starvation, resulting in Rim15 nuclear localization and induction of the G0 program. These findings identify a novel function for autophagy genes in mediating cell cycle arrest during nutrient stress, and a novel function for Atg6 in the selective targeting of proteins for autophagic degradation. These observations may help to explain how the metazoan orthologs of ATG6 and other yeast autophagy genes function in development, cell cycle regulation, and tumor suppression.

402/B349

A Novel PLZF Isoform (p65) Is Produced by an Alternative Signal of Translational Initiation.

C. Jones, I. Fréchette, F. Boudreau; Anatomie et biologie cellulaire, Université de Sherbrooke, Sherbrooke, QC, Canada

PLZF is a transcriptional repressor known to be implicated in the maintenance of spermatogonial stem cells. Stem cells are essentials for the maintenance of many other systems such as the intestinal epithelium that is rapidly and constantly renewed. We have previously identified PLZF as a direct transcriptional target of the CUX1 protein. In order to gain a better comprehension of the role of PLZF in intestinal homeostasis, we first verified the expression of PLZF in intestinal and colonic epithelial cell lines. A Western blot analysis with protein extracts from colorectal cancer cell lines and normal intestinal epithelial cells showed the expression of a faster migrating protein (65 KDa) in comparison to the classic form of PLZF (81 KDa) that was not detectable in these cell lines. A detailed analysis of the PLZF coding sequence allowed us to identify an internal initiation translation site with a consensus Kozak sequence that was evolutionary conserved. To confirm the implication of this site in the production of PLZF 65 KDa (p65), we proceeded to an in vitro transcription/translation assay with an expression vector containing a modified version of PLZF, in which we removed the classical initiation site. This assay led to the production of p65. The transfection of the same construct in 293T cells produced of a protein of the same molecular weight than the protein detected in intestinal epithelial cells. This was further confirmed by immunoprecipitation experiments. Fractionation of cell compartments and immunofluorescence with colorectal cancer cells showed restricted cytoskeleton localization of p65. A Western blot analysis with protein extracts from Caco-2/15 cells at different confluence levels showed a drastic diminution of p65 expression with post-confluence. Synchronization of normal intestinal epithelial cells with serum deprivation revealed a rapid induction of p65 following re-entry of the cell cycle. This induction declined overtime during the progression of the cell cycle. Overall, these results suggest that PLZF p65 origins from an internal initiation translation site and could play a role during the GO/G1 phase of the cell cycle in intestinal epithelial cells.

403/B350

Serum Starvation Induces G1 Arrest through Suppression of Skp2-CDK2 and CDK4 in SK-OV-3 Cells.

J. Shin[1,2], S. Lee[1], J. Park[1], D. Jin[1], M. Lee[1]; [1]Biology, Sookmyung Women's University, Seoul, South Korea, [2]Biology, Konkuk University, Seoul, South Korea

Recent studies have suggested that Skp2, an SCF-type ubiquitin ligase, positively regulates cell cycle through degradation of p27, which is an inhibitor of cyclin-dependent kinase 2 (CDK2), which drive cells from the G1 to S phase of cell cycles. In the present study, we examined key regulatory proteins involved in serum starvation-induced cell cycle arrest in human ovarian cancer cells, SK-OV-3. Cell cycle analysis showed that cells were arrested at the G1 phase after serum starvation. Western blot analysis showed that the protein levels of CDK4 and

CDK2 were significantly decreased in SK-OV-3 cells. Consistently, Roscovitine, an inhibitor of CDK2, induced cell cycle arrest in normally proliferating cells and a chemical inhibitor of CDK4, 3-ATA [3-Amino-9-thio(10H)-acridone], was found to induce growth arrest. We also found that the protein level of Skp2 was dramatically decreased in response to serum starvation. Moreover, CDK2 protein, which allows cell cycle transit from the G1 to the S phase, was decreased when the Skp2 expression was inhibited by specific siRNA of Skp2, but CDK4 was not decreased. Therefore, these results suggest that serum starvation induces G1 arrest through suppression of Skp2-dependent CDK2 activity and Skp2-independent CDK4 activity in human ovarian cancer cells, SK-OV-3.

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A Novel Pathway Required for Regulation of G1 Cyclin Levels via the PP2A Rts1 Phosphatase.

K. Artiles, D. McCusker, S. Anastasia, D. Kellogg; Department of Molecular, Cell and Developmental Biology, UCSC, Santa Cruz, CA

Cell cycle entry is a crucial event that is triggered by transcription of G1 cyclins. In budding yeast, G1 cyclins promote early cell cycle events such as polarized cell growth and bud formation. Here, we show that a regulatory subunit of the PP2A phosphatase, Rts1, plays a role in mechanisms required for G1 events. Loss of Rts1 caused reduced and delayed accumulation of the G1 cyclin Cln2, and a corresponding delay in G1 events. Loss of Rts1 also resulted in reduced polar cell growth, and *rts1*Δ showed strong genetic interactions with G1 cyclins. A number of experiments indicate that Rts1 does not act solely in pathways required for transcription of Cln2. In addition, Rts1 is not required for SCF-dependent destruction of Cln2 and does not appear to regulate the stability of the Cln2 protein. Together, these observations suggest that Rts1 regulates levels of the Cln2 protein or mRNA via novel post-transcriptional mechanisms. The Cln2 protein is hyperphosphorylated in *rts1*Δ cells, which suggests that $PP2A^{Rts1}$ may act directly on Cln2. Rts1 may be a component of a recently discovered but poorly understood positive feedback loop that regulates accumulation of G1 cyclins and entry into G1.

405/B352

The Activity of the S. cerevisiae G1 Cyclin Cln3 Is Dependent on THI73.

J. G. Hancock, M. Miller; Biology, Rhodes College, Memphis, TN

The cell cycle is the highly regulated process by which a cell grows, replicates, and divides into two identical daughter cells. In *Saccharomyces cerevisiae*, cell cycle initiation or passage through "Start" is regulated by the G1 cyclin Cln3. During G1, Cln3 binds to and activates the cyclin-dependent kinase, Cdc28. The Cln3-Cdc28 complex functions to support cell cycle progression by allowing the transcription of a subset of genes required for subsequent cell cycle events. Study of the Cln3 pathway in yeast is of particular interest due to its functional similarity to the Retinoblastoma tumor suppressor pathway in mammalian cells. Activity of the Cln3-Cdc28 complex is provisional on its Ran-dependent movement into the nucleus. A C-terminal bipartite type nuclear localization signal (NLS) is known to be required for proper nuclear import. Using a Green Fluorescent Protein (GFP) reporter system, nineteen of seventy-nine genes tested were identified as important for the ability of the Cln3 NLS to support movement of GFP into the nucleus. Of these nineteen, nine have been found to show full length Cln3 functional defects. One of these nine genes, *THI73*, is induced under low thiamine (Vitamin B) conditions, and implicated as a membrane bound transporter based solely on sequence analysis. Data gained from multiple viability assays support the idea that *THI73* is important for full length Cln3-dependent viability. Cells containing a *thi73* deletion show approximately a ten-fold decrease in viability compared to cells containing *THI73* when growth is dependent on Cln3 function. Additionally, we observed that a monopartite SV40 NLS and a bipartite nucleoplasmin NLS were both able to function in the absence of *THI73*, in contrast to our results using the Cln3 NLS. This suggests some specificity of action between Thi73 and Cln3. Since in the absence of *THI73* we observe both a defect in Cln3 NLS-GFP activity and decrease in Cln3-dependent viability, we hypothesize that the reason that Cln3 is not functioning appropriately is because Thi73 impacts the ability of Cln3 to enter the nucleus.

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Regulation of the Cell Cycle on Progression in A549 Human Lung Cell and MCF-7 Human Breast Cell Treatment by Brazilian Medicinal Plant.

H. Tokuda[1], M. Kaneko[2], M. Yamashita[2], A. Iida[3]; [1]Molecular Biochemistry, Kyoto Prefectural University of Medicine, Kyoto, Japan, [2]Pharmacology, Takasaki University of Welfare and Health, Takasaki, Japan, [3]Agriculture, Kinki University, Nara, Japan

Tabebuia avellanedae (TA), which is a plant that has been used for herbal medicine in South America and from Brazil to northern Argentina, is well known in traditional folk medicne used for the treatment of various diseases during five hundred years. The inner bark of this plant produced in Brazil is distributed in Asia as a herb tea and healthy life purpose. Previously, we reported that extract essense of TA (TA ess.) and including naphthoquinones type compounds (NQ 801) inhibited TPA-induced in vitro conventional assay and thereby acted as a chemopreventive agents against carcinogenic compounds, using mouse experimental systems. Furthermore, NQ801 had potent growth inhibitory effects in vitro against A549 and MCF-7 cells under usual condition. In this study, the purpose of more detail analysis of this useful sample was to develop the cell cyclic phase studies as an effective cancer chemosensitivity test and cell cycle was investigated by western blotting analysis. The antiproliferative effects of A549 and MCF-7 by NQ801 were found to be accompanied by a G1 arrest on cell cycle, following the expressions of cyclin D1 and cdk2. This notion was further supported by examination of other cell cycle-dependent expression and these results suggest that growth arrest induced by NQ801 blocks both cells in G1 phase of the cell cycle. Based on our in vitro and in vivo observation, it is tempting to speculate that NQ801 may find its place as interesting agent for the prevention and/or treatment human derived cancer cells.

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Versatile Roles of a Splice-generated C-terminal Extension of hCenexin1 in Polo-like Kinase 1 (Plk1)-dependent Mitotic Functions and Plk1-independent Ninein Recruitment and Ciliogenesis.

N. Soung[1], L. Yu[2], J. Park[1], K. H. Lee[1], J. Lee[3], T. Veenstra[4], K. Rhee[3], K. S. Lee[1]; [1]Metabolism, National Institutes of Health, Bethesda, MD, [2]National Center for Toxicological Research, U.S. Food and Drug Administration, Jefferson, AR, [3]School of Biological Sciences, Seoul

National University, Seoul, South Korea, [4]Laboratory of Proteomics and Analytical Technologies, National Cancer Institute-Frederick, Frederick, MD

Outer Dense Fiber 2 (ODF2) was initially identified as a major component of sperm tail cytoskeleton, and was later suggested to be a centrosomal scaffold important for the recruitment of various proteins to somatic centrosomes. Here, we showed that a splicing variant of hODF2 called hCenexin1, but not hODF2, was abundantly associated with centrosomes in somatic cells and tissues. Unlike hODF2, hCenexin1 possesses a unique C-terminal extension that is vital for proper localization to the mother centriole and interaction with a mitotic polo-like kinase 1 (Plk1) through a Cdc2-dependent phospho-S796 motif within the C-terminal extension. The hCenexin1-Plk1 interaction was critical for proper bipolar spindle formation during mitosis. Likewise, overexpression of hCenexin1, but not hCenexin1(S796A), induced ectopic hCenexin1-Plk1 assemblies capable of both recruiting Kendrin and γ-tubulin in a Plk1 activity-dependent manner and forming ectopic microtubule organizing centers. Early in the cell cycle, hCenexin1, but not hODF2, also contributed to normal ninein recruitment and primary cilia formation but independently of Plk1 binding. These findings provide an unparalleled example of how a C-terminal extension of a splicing variant of a sperm tail-associating protein can mediate unanticipated phospho-dependent and -independent roles for distinct cellular processes at somatic centrosomes.

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Identification of a Novel Aurora-A Substrate, PUM2, Promotes Aurora-A Kinase Activity and Protein Stability.

Y. Huang[1], C. Chou[2], C. F. Huang[1]; [1]Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan, [2]Department of Life Science, Chang-Gung University, Tao-Yuan, Taiwan

Aurora-A, a centrosomal serine-threonine kinase, orchestrates several key aspects of cell division, such as centrosome maturation, spindle assembly, mitotic commitment and cytokinesis. However, the regulatory pathways for Aurora-A are still controversial. In this study, we demonstrate a RNA-binding protein, PUM2, is a novel substrate and interacting protein of Aurora-A. Instead of using well-characterized mechanism of translational repression, PUM2 is involved in Aurora-A regulation by physical interaction. Overexpression of PUM2 causes an increase of Aurora-A half-life, whereas PUM2 depletion results in down-regulation of both PUM2 and Aurora-A. PUM2 binds to APC/Cdh1 recognition domain, A-box, of Aurora-A, and this association is required for maintaining Aurora-A protein stability and enhancing its kinase activity. Aurora-A is less susceptible to ubiquitination in cells overexpressed with PUM2, suggesting that binding of PUM2 might protect Aurora-A from being attack by APC/Cdh1-mediated degradation. Moreover, PUM2 and Aurora-A could affect cell cycle progression and arrests cells at G2/M phase synergistically. We propose a model in which the proper phosphorylation and accumulation of PUM2 at the centrosome might function as a scaffold for the recruitment of other Aurora-A activator, which might trigger the increase of Aurora-A activity dramatically and cause mitotic commitment.

409/B356

The Drosophila Homolog of MCPH1, a Human Microcephaly Gene, Is Required for Genomic Stability in the Early Embryo.

J. Rickmyre, L. A. Lee; Cell & Developmental Biology, Vanderbilt University Medical Center, Nashville, TN

Mutations in human *MCPH1* result in a form of autosomal recessive primary microcephaly, a disorder of fetal-brain growth characterized by a severely reduced cerebral cortex and head size with mental retardation. Both human and *Drosophila* MCPH1 contain BRCA1 C-terminal domains (BRCT domains), which are found in many proteins that function in DNA repair and cell-cycle control. Previously, we demonstrated that maternal-effect lethal mutations in *Drosophila mcph1* result in cell-cycle arrest due to triggering of the centrosome inactivation pathway, a Checkpoint kinase 2 (Chk2)-mediated response following DNA damage or incomplete DNA replication in early *Drosophila embryos*. *mcph1*-derived embryos exhibit genomic instability as evidenced by frequent chromatin bridging in anaphase. Furthermore, in contrast to studies of human *MCPH1*, our data indicate that the ATR/Chk1-mediated DNA checkpoint is intact in Drosophila *mcph1* mutants. Components of this checkpoint, however, appear to cooperate with MCPH1 to regulate embryonic cell cycles in a manner independent of Cdk1 phosphorylation. Finally, in collaboration with Scott Waddell (UMass Worcester), we demonstrated that brains of *mcph1* adult male flies display defects in the mushroom bodies, a neuronal structure important for learning and memory, suggesting an evolutionarily conserved role for MCPH1 in brain development. Current efforts are directed toward placing MCPH1 within a molecular framework using a tandem affinity purification-mass spectrometry approach.

410/B357

Cell Cycle Related Kinase (CCRK) Is Required for Centrosome Duplication in Colon Cancer Cells.

X. An, S. S. Ng, H. Kung, M. C. Lin; Chemistry, The University of Hong Kong, Hong Kong, China

Background & Aims: Cell cycle related kinase (CCRK) is a newly identified cdk-activating kinase (CAK) homologous to Cdk7. The aim of this study was to investigate the role of CCRK in centrosome duplication in colon cancer cells. Methods: We designed small interfering RNAs targeting CCRK (siCCRK) and examined cell cycle progression, centrosome duplication, and mitotic catastrophe in colon cancer cell lines. Results: Suppression of CCRK by siCCRK could significantly inhibit the growth of the colon cancer cells. Flow cytometry analysis demonstrated that CCRK knock-down resulted in G1 and G2-M phase arrests, induced mitotic catastrophe and suppressed centrosome duplication, which were accompanied by downregulation in cyclin E, phosphorylated Cdk2, and phosphorylated Rb. Conclusions: CCRK is crucial for cell cycle progression and is necessary for centrosome duplication in colon cancer cells.

411/B358

Distinctive Role of Akt1/PKBα in G1/S Transition and Proliferation.

S. Yun, E. Kim, M. Kim, K. Do, J. Ha, S. Lee, C. Kim, S. Bae; Pharmacology, School of Medicine, Pusan National University, Busan, South Korea

Phosphatidylinositol 3-kinase (PI3K) pathways play key regulatory roles in the cell cycle progression into S phase. In this study, we examined the regulation of Akt/protein kinase B (Akt/PKB) isoforms during G1/S transition in synchronized mouse embryo fibroblast (MEF) cells. MEF cells lacking Akt1/PKBα resulted in 70% decrease of proliferation whereas cells lacking Akt2/PKBβ did not show significant

difference from that of wild type cells. Moreover, re-expression of Akt1/PKBα completely restored the proliferation of double Akt1/PKBα and Akt2/PKBβ knock-out (DKO) cells. Analysis of cell cycle revealed that initiation of G1/S transition of cells lacking Akt1/PKBα was delayed about 4 hrs compared with wild type and cells lacking Akt2/PKBβ. Delayed G1/S transition in DKO cells was restored by forced expression of Akt1/PKBα. However, stimulatory effect of Akt1/PKBα on G1/S transition and proliferation was abolished by swapping pleckstrin homology (PH) domain with that of Akt2/PKBβ. Addition of serum to the synchronized cells evoked long lasting phosphorylation at Ser473 of Akt1/PKBα. In contrast, the Ser474 phosphorylation of Akt2/PKBβ was rapid and transient compared with Akt1/PKBα. Moreover, replacing PH domain of Akt1/PKBα with that of Akt2/PKBβ resulted in the loss of Ser473 phosphorylation. Finally, DKO cells showed defects in cyclin D1 induction as well as Rb phosphorylation, which was restored by forced expression of Akt1/PKBα. Given these results, we suggest that PH domain of Akt1/PKBα provides sustained kinase activity which may be necessary for G1/S transition and cell cycle progression.

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The Identification of Cyclin A Interacting Proteins in S and G2 Phases.

F. J. Walton[1], M. Collins[2], J. Choudhary[2], J. Pines[1]; [1]Gurdon Institute, Cambridge University, Cambridge, United Kingdom, [2]The Wellcome Trust Sanger Institute, Wellcome Trust Genome Campus, Hinxton, United Kingdom

The key to regulation of the human cell cycle is the activity of the cyclin-dependent kinases determined by their activating partners, the cyclins. Of particular interest are cyclins A and B as they are the only cyclins that have an essential role in cell proliferation in mammalian knockout models. Cyclin B promotes entry into and progression through mitosis, wheras cyclin A functions during both S and G2 phases. While a number of regulators and substrates of cyclin A are known, these interactions do not provide a clear mechanism to explain why cyclin A is essential for completing S and G2 phases. To address this problem, cyclin A complexes have been isolated from total cell extracts of synchronized HeLa or hTERT-RPE1 cell lines via immunoprecipitation. Analysing the cyclin A complexes from HeLa cells by mass spectrometry revealed 187 proteins specifically coimmunoprecipitating with cyclin A. Many previously known cyclin A interaction partners, including Cdk1, Cdk2, p21, Cdc20, Cks1 and Skp2, were identified. The additional proteins identified can be classified as involved in cytoskeletal regulation, DNA replication or repair, intracellular trafficking including endocytosis and exocytosis, ubiquitination and protein degradation. Furthermore, twenty-nine interactors are poorly characterized or have not previously been noted to have a role in cell cycle progression. These results and future characterization of these novel interactions will help elucidate the mechanisms of cyclin A function through the human cell cycle.

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Possible Involvement of the Salvador-Warts-Hippo Pathway in the p53 Response to Cytoskeletal Disruption.

G. Montano, L. Wilmeth, C. Shuster; Biology, New Mexico State University, Las Cruces, NM

The NDR family of protein kinases have been shown to participate in different cellular pathways all related to the control of cell proliferation and apoptosis. In yeast, Dbf2/Sid2 kinases play critical roles in the coordination of mitosis and cytokinesis, whereas in animal cells, Drosophila Warts and human Lats1/2 kinases negatively regulate cell proliferation through the Salvador-Warts-Hippo (SWH) pathway. In all cases, these kinases require the association of a small regulatory subunit (Mob1) for proper kinase activation and function. It has also been shown that Lats2 is required for the p53 response to chronic nocodazole treatment (Genes Dev 20: 2687-2700), although it is unclear whether the other components of SWH pathway are similarly required. We identified Lats2 in a yeast two-hybrid screen for proteins that interact with Mob1A and found that Lats2 interacted solely with Mob1A, but not with other Mob1 isoforms. Further, introduction of missense mutations corresponding to nonfunctional alleles in yeast Mob1 ablated Mob1-Lats2 interactions. To investigate whether Mob1 or other elements of the of SWH pathway are required for p53 stabilization following cytoskeletal disruption, human RPE1 cells were depleted of Lats2, Mob1A, and Mst2 (Hippo), treated with nocodazole, and assayed for p53 stabilization. Whereas cells transfected with non-targeting siRNAs demonstrated an elevation of p53 within hours of drug addition, cells depleted of Lats2, Mob1 and Mst2 all failed to sustain a p53 response relative to controls. Together, these results suggest that the SWH pathway may play a role in stress responses in addition to its previously described roles in regulating cell proliferation during development. Current efforts are focused on a series of add-back experiments where wild-type as well as Lats2-binding and Mst2 phosphorylation site mutants of Mob1 will be reintroduced into Mob1-depleted cells, and assayed for their ability to rescue the p53 response.

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Regulation of HsMis13 Complex Assembly Dynamics and Plasticity by Aurora B.

Y. Yang[1,2], T. Ward[1,2], F. Yan[1,2], X. Ding[1,3], X. Yao[1]; [1]Cellular Dynamics, University of Science & Technology of China, Hefei, China, [2]Physiology, Morehouse School of Medicine, Atlanta, GA, [3]Medicine, Beijing University of Chinese Medicine, Beijing, China

Chromosome movements in mitosis are orchestrated by dynamic interactions between spindle microtubules and the kinetochore, a multi-protein complex assembled onto centromeric DNA of the chromosome. Here we show that phosphorylation of human HsMis13 by aurora B kinase is required for functional kinetochore assembly in HeLa cells. Aurora B interacts with HsMis13 in vitro and in vivo. HsMis13 is a cognate substrate of Aurora B and the phosphorylation sites were mapped to Ser100 and Ser109. Suppression of Aurora B kinase by either small interfering RNA or chemical inhibitors abrogates the localization of HsMis13 but not HsMis12 to the kinetochore. In addition, non-phosphorylatable but not wild type and phospho-mimicking HsMis13, failed to localize to the kinetochore, demonstrating the requirement of phosphorylation by aurora B for the assembly of HsMis13 to kinetochore. In fact, localization of HsMis13 to the kinetochore is spatiotemporally regulated by aurora B kinase, which is essential for recruiting outer kinetochore components such as Ndc80 components and CENP-E for functional kinetochore assembly. Importantly, phospho-mimicking mutant HsMis13 restores the assembly of CENP-E to the kinetochore and tension developed across the sister kinetochores in aurora B-inhibited cells. To assess the assembly dynamics of HsMis13 complex, we adopted a new covalent fluorescent pulse-chase labeling approach using SNAP tagging. Assembly of new HsMis13 complex is achieved in prophase and regulated by aurora B activity. Our data suggest that HsMis13 phosphorylation by aurora B is required for assembly of HsMis13 complex to the kinetochore and organizing a stable bi-oriented microtubule kinetochore attachment that is essential for

faithful chromosome segregation in mitosis. Currently, we are analyzing how HsMis13 phosphorylation dynamics are regulated during chromosome segregation in real-time mitosis.

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Coordination of rDNA Segregation, Spindle Elongation, and Cytokinesis.

B. Zipp, K. Kaplan; Molecular and Cellular Biology, UC Davis, Davis, CA

To maintain mitotic fidelity, chromosome segregation must be completed before spindle breakdown and cytokinesis. In budding yeast, rDNA repeats on chromosome XII segregate last during anaphase. Segregation of rDNA depends both on the actions of the mitotic spindle and on chromosome condensation machinery. However, it is unclear how spindle movement and chromosome structure are coordinated to ensure rDNA is faithfully segregated before cytokinesis. Interestingly, the Aurora B kinase homologue, Ipl1, has been implicated in condensation of chromosome XII, spindle elongation and cytokinesis. We propose that Ipl1 complexes are required to coordinate these anaphase events and participate in a feedback loop to ensure proper chromosome segregation. To test this model, we have used live cell imaging to ask if changes in rDNA structure affect spindle elongation and cytokinesis. We observed that acute hydroxyurea treatment leads to the formation of rDNA bridges as visualized by Nop1-GFP, delayed segregation of rDNA, as well as slowed spindle elongation. In contrast, the Ipl1-321 mutant that inhibits rDNA condensation, accelerates rDNA segregation towards the spindle poles. We have also observed a temporal correlation between complete rDNA segregation, spindle breakdown, and abscission. These results indicate that rDNA provides negative feedback on spindle elongation and abscission. We therefore propose a model where rDNA regulates anaphase and cytokinesis to ensure mitotic fidelity.

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Perturbation of the Chromosomal Binding of RCC1, Mad2 and Survivin Causes Spindle Assembly Defects and Mitotic Catastrophe.

C. Ho, C. Wong, H. Li; School of Biological Sciences, Nanyang Technological University, Singapore, Singapore

Mitotic catastrophe is a form of cell death that results from aberrant mitosis. Currently, the mechanisms involved in this form of cell death remain poorly understood. We found that actinomycin D induces mitotic catastrophe with severe spindle assembly defects. We have studied the nature of three groups of chromosome binding proteins in mitotic cells treated with actinomycin D. We found that actinomycin D reduced the binding affinity of RCC1 to the mitotic chromosome, which led to a reduction of RanGTP level. In addition, Mad2 was not concentrated at the kinetochores, indicating that the mitotic spindle checkpoint was affected. Furthermore, the localization of survivin was altered in cells. These data suggested that chromosomal binding of the mitotic regulators such as RCC1, Mad2 and survivin is essential for mitotic progression. Mitotic chromosomes not only carry the genetic material needed for the newly synthesized daughter cells, but also serve as docking sites for some of the mitotic regulators. Perturbation of their binding to the mitotic chromosome by actinomycin D could affect their functions in regulating mitotic progression thus leading to severe spindle defects and mitotic catastrophe

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Polyubiquitination Determinants on Ubc1, an APC-dependent E2.

M. C. Rodrigo Brenni, D. O. Morgan; Physiology and Biochemistry and Biophysics, University of California, San Francisco, San Francisco, CA

The Anaphase-Promoting Complex (APC) is an E3 ubiquitin ligase that assembles polyubiquitin chains on substrates important for cell cycle progression. In budding yeast, the APC collaborates with two E2 ubiquitin-conjugating enzymes, Ubc4 and Ubc1. We have demonstrated that Ubc4 and Ubc1 have very different enzymatic behaviors: APC reactions with Ubc4 result in rapid monoubiquitination of multiple nonspecific lysines on the substrate, whereas APC reactions with Ubc1 result in the assembly of polyubiquitin chains that are linked specifically through lysine 48 (K48) of ubiquitin. We are now mapping the surfaces on Ubc1 that are responsible for its K48 linkage specificity. We focused on two clusters of amino acids that are found on the protein surface near the catalytic cysteine of Ubc1 but are not present in Ubc4. Changing these amino acids in Ubc1 to those in Ubc4 resulted in a specific defect in K48-linked ubiquitination activity. Nonspecific Ubc1 activity toward other lysines was not affected. Further analysis revealed that the K48-linked ubiquitination activity depends on a single amino acid near Ubc1's catalytic cysteine. We therefore speculate that this amino acid in Ubc1 helps guide K48 of the attacking ubiquitin toward the catalytic cysteine. Although this residue is required for K48-linked ubiquitination by Ubc1, it is not sufficient to confer this activity on Ubc4, since mutation of the same cluster on Ubc4 to those found in Ubc1 does not result in K48-linked chains. We are now identifying residues in the attacking ubiquitin that interact with Ubc1 and confer K48-linked ubiquitination activity.

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Chk1 Phosphorylation by Cyclin-dependent Kinase 1 Promotes Mitotic Entry.

H. Goto[1], M. Enomoto[1,2], Y. Tomono[3], K. Kasahara[1], Y. Ikegami[1,4], K. Tsujimura[5], T. Kiyono[6], M. Inagaki[1,2]; [1]Division of Biochemistry, Aichi Cancer Center Research Institute, Nagoya, Japan, [2]Department of Cellular Oncology, Nagoya University Graduate School of Medicine, Nagoya, Japan, [3]Division of Molecular and Cell Biology, Shigei Medical Research Institute, Okayama, Japan, [4]Department of Nephro-urology, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan, [5]Department of Microbiology and Immunology, Hamamatsu University School of Medicine, Hamamatsu, Japan, [6]Virology Division, National Cancer Center Research Institute, Tokyo, Japan

Chk1, one of critical transducers in DNA damage/replication checkpoint, prevents entry into mitosis through the inhibition of Cyclin-dependent kinase 1 (Cdk1) activity. Chk1 is phosphorylated at Ser286 and Ser301 by Cdk1 in mitosis, but its functional significance remains unknown. Here, we show that Chk1 was translocated from the nucleus to the cytoplasm in prophase, the first mitotic phase at which chromosomes are condensed but nuclear membrane remains intact. Exogenous Chk1 mutated at Ser286 and Ser301 to Ala (S286A/S301A) was mainly observed at the nucleus in prophase cells although such nuclear accumulation was hardly observed in the case of Chk1 wild type (WT). Induction of S286A/S301A results in dramatic delay of mitotic entry, compared with WT case. The phospho-mimic mutant S286E/S301E had opposite effects not only on the location in prophase but also on the mitotic entry. In addition, K38M/S286A/S301A mutant, a kinase dead-version of S286A/S301A had no effect on mitotic delay although it was also accumulated in the nucleus like

S286A/S301A. These results indicated that Cdk1 plays a critical role in cytoplasmic sequestration of Chk1 activity through direct phosphorylation reaction at G2/M transition and this nuclear elimination of Chk1 activity is one of key signaling pathways for controlling the proper order of mitotic entry.

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Polo-like Kinase 1 Promotes Mitotic Entry through Associating with Its Spatiotemporal Regulator Calmodulin in Mammalian Cells.

C. Li[1,2,3], G. Dai[4], S. Wang[1]; [1]Medical School, Nanjing University, Nanjing, China, [2]Life Science College, Nanjing Normal University, Nanjing, China, [3]Model Animal Research Center (MARC), Nanjing University, Nanjing, China, [4]Department of Biology, Jiangsu Institute of Education, Nanjing, China

Cell cycle control is a process requiring, the presence of, not only the right protein at the right time, but also the right protein at the right place. Both Polo-like kinase (Plk1) and CaM are such cell cycle regulators whose activity and localization are temporally and spatially controlled. FRET analysis in living cells using CFP-Plk1 and YFP-CaM as probes shows that Plk1 and CaM interact in a distinct spatiotemporal pattern from late G2 to metaphase when they colocalize at the centrosome and spindle poles. In vivo FRET and in vitro binding experiments suggest that CaM can associate with CaM binding motifs located at the kinase and polo box domain of Plk1, greatly stimulating its kinase activity at late G2 phase. The cooperation of CaM and Plk1 is able to hyperphosphorylate Cdc25C and thus promote mitotic entry. We hypothesize that Plk1 activity could be temporally and spatially regulated by CaM binding and then phosphorylate Cdc25C to drive the cell cycle transition.

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Role of Cdc14 Proteins in Vertebrate Cells.

A. Mocciaro[1], K. Zeng[2], P. Vagnarelli[3], W. Earnshaw[3], E. Schiebel[1]; [1]ZMBH- University of Heidelberg, Heidelberg, Germany, [2]Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom, [3]Wellcome Trust Centre for Cell Biology,Institute of Cell and Molecular Biology, University of Edinburgh, Edinburgh, United Kingdom

The budding yeast Cdc14 is the prototype of a family of highly conserved protein phosphatases that are present from yeast to humans and have important functions in cell cycle regulation. The budding yeast Cdc14 controls mitotic exit, anaphase spindle formation, cytokinesis and rDNA segregation. Relatively little is known about the two vertebrate Cdc14 phosphatases, Cdc14A and Cdc14B. RNA interference data suggest that human Cdc14A has an ill-defined role in centrosome duplication and cytokinesis, while human Cdc14B seems to be involved in regulation of centrioles duplication and in the G2 DNA-damage-response checkpoint. We are currently investigating the role of Cdc14 proteins in vertebrates, using chicken DT40 B-Lymphoma cells. DT40 cells have a very high level of homologous recombination and therefore allow the disruption of genes with high efficiency. Our analysis of the chicken genome identified a homologue of hCdc14A, called cCdc14A, and a homologue of hCdc14B, called cCdc14B. Analysis of cCdc14A localization by indirect immunofluorescence showed that the protein is associated with centrosomes in interphase, but it leaves the centrosomes in prophase and is cytoplasmic throughout mitosis, like its human homologue hCdc14A. Overexpression of cCdc14A leads to centrosomes overduplication, mitotic spindle and chromosome segregation defects and a failure to complete cytokinesis. We generated a knock out cell line for cCdc14A and we observed that cCdc14A-/- cells are viable; this is the first evidence that Cdc14A is not essential in vertebrate cells. We next generated cell lines in which cCdc14B gene has been disrupted; cells lacking cCdc14B are viable and they do not show significant alterations in the cell cycle. Using indirect immunofluorescence and live cell imaging we observed that cCdc14B KO cells show a small delay in sister chromatids separation during anaphase, but this does not significantly affect the overall duration of mitosis. To further investigate the different functions of the two Cdc14 phosphatases during cell cycle and to discriminate if one protein is partially fulfilling the role of the other, we are currently working on generating a cell line disrupted for both cCdc14A and cCdc14B.

421/B368

Chromosomal Passenger Proteins Coordinate Spindle Disassembly with Septin Dynamics.

D. Rozelle, K. Kaplan; Molecular and Cellular Biology, UC Davis, Davis, CA

Regulatory checkpoints and feedback pathways play an important role in ensuring the proper order of mitotic events. Chromosome passengers dramatically redistribute to the anaphase spindle and have been suggested to coordinate chromosome segregation with mitotic exit. Our recent observations in budding yeast have demonstrated chromosome passengers (including CBF3, Bir1, Sli15 and Ipl1) redistribute to the anaphase spindle and regulate both spindle elongation and septin dynamics. We propose a model whereby chromosome passengers on the spindle are restrained from acting on septins until after spindle breakdown. Using live-cell imaging, we have found that anaphase spindle breakdown precedes septin ring dynamics (septin separation and disassembly) and disassembly requires a Sli15p-Bir1p complex. Prior to spindle breakdown, FRAP experiments show that chromosome passengers stably associate with the spindle, consistent with the idea that they are restrained from acting elsewhere. As a further test of this possibility, we prematurely induced spindle breakdown (i.e., MAD2Δ + nocodazole) and observed a dramatic acceleration of septin disassembly. We suggest that rapid disassembly of septin rings prevent cytokinesis and results in multi-budded cells. The accelerated disassembly of septins is dependent on chromosome passengers, consistent with them acting downstream of spindle disassembly. Indeed, chromosome-passenger mutants delay septin disassembly and restore cytokinesis in cells lacking an anaphase spindle. As there is no evidence of chromosome passengers' direct interaction with septin filaments, we propose that they indirectly affect the modification of septins during anaphase. Consistent with this possibility, we observed that chromosome-passenger mutants alter the timing of septin sumoylation. Our work will now focus on identifying the key interactions that link chromosome passengers to septin modification and to changes in septin dynamics.

S/DNA Replication and Forward (422-440)

422/B369

Molecular Nature of a Novel Checkpoint for Monitoring Activation of Replication Origins.

S. Tudzarova[1], M. W. Trotter[2], C. Malvey[3], J. Godovac-Zimmermann[3], K. Stoeber[1], G. Williams[1]; [1]Research Department of Pathology, Wolfson Institute for Biomedical Research and UCL Cancer Institute, University College London, London, United Kingdom, [2]Cambridge Institute for Medical Research, University of Cambridge, London, United Kingdom, [3]Department of Medicine, The Rayne Institute and Centre for Molecular Medicine, University College London, London, United Kingdom

The DNA replication licensing machinery has recently emerged as a promising target for cancer diagnosis and therapy. The Dbf4-dependent kinase Cdc7 (DDK) plays an essential role in the firing of replication origins by phosphorylating and activating the helicase Mcm2-7 leading to unwinding of the DNA helix and recruitment of the replisome. Inhibition of Cdc7 activity by siRNA knockdown or small molecule kinase inhibitors induces apoptotic cell death in cancer cells, with untransformed cells arresting cell cycle progression in G1 phase. This cancer cell specific killing points towards a novel 'licensing checkpoint' which monitors activation of a critical number of replication origins, and that is inactivated or lost in cancer cells. We have used siRNA knockdown to deplete Cdc7 in untransformed IMR-90 lung fibroblasts, and applied a combined proteomics, transcriptomics and cell biology approach to functionally characterise the molecular workings of the putative licensing checkpoint. Our data show that the checkpoint is p53-dependent, and that p53 negatively regulates two proliferation-associated signalling pathways (details of which will be presented) which ultimately leads to the inhibition of S-phase inducing CDKs and arrest in G1 phase. Importantly IMR-90 cells arresting in G1 phase upon activation of this checkpoint remain viable and resume DNA synthesis when the block to origin firing is removed. Taken together our data support the concept of the origin licensing machinery as a promising non-genotoxic therapeutic target. An understanding of the signalling pathways involved in the molecular workings of the licensing checkpoint will be important for selecting patient groups that are likely to respond to this new form of treatment

423/B370

Functional Roles of Two Human Rad5 Homologs in Preventing Damage-induced Mutagenesis during DNA Replication.

J. Lin, K. Cimprich; Chemical and Systems Biology, Stanford University, Stanford, CA

Post-replication repair maintains genomic stability by allowing bypass of replication forks stalled by DNA lesions. Because lesion bypass can occur through either an error-prone or error-free pathway, lesion bypass activity must be tightly regulated to prevent mutagenesis. Studies in yeast indicate Rad5 plays an important role in error-free lesion bypass, and recently, two human Rad5 homologs, SHPRH and HLTF, have been identified. While both of these E3-ligases have been shown to promote PCNA polyubiquitination, the functional roles of SHPRH and HLTF in post-replication repair remain unclear. Here we demonstrate that both SHPRH and HLTF are required for preventing damage-induced mutagenesis. We also show that the effect of SHPRH and HLTF on mutation suppression differs with the type of DNA damage induced. In order to investigate the damage specificity of these two Rad5 homologs, we analyzed their chromatin recruitment and their interaction with Rad18, the E3-ligase specific for PCNA monoubiquitination and an important regulator of error-prone lesion bypass. Although the chromatin binding properties of SHPRH and HLTF are not affected by DNA damage, the interaction between Rad18 and SHPRH/HLTF shows a damage-specific pattern. Our results suggest that SHPRH and HLTF mediate different branches of error-free post-replication repair for different DNA damaging agents. The increased complexity of lesion bypass in higher eukaryotes might help to prevent mutagenesis and to maintain genomic stability.

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Cyclin D1 Is Phosphorylated by ATM and ATR as Part of the G1/S Phase DNA Damage Checkpoint.

D. Stacey, K. Yang, A. W. Stacey, M. Hitomi; Molecular Genetics, The Cleveland Clinic Found., Cleveland, OH

We have used single cell analytical techniques to identify a role for DNA damage checkpoint kinases in normal cell cycle control. Microinjection and quantitative image analysis were used to demonstrate that cyclin D1 levels decline during S phase due to phosphorylation of Thr 286. This decline is essential to DNA synthesis, and to the overall regulation of cell cycle progression. Despite its importance, however, we were unable to confirm reports identifying the kinase or regulatory signaling pathways involved in Thr 286 phosphorylation. Rather, our evidence suggested that DNA synthesis itself might regulate this phosphorylation. To test this idea, ATR activity was induced by single stranded DNA breakage following UV irradiation, or by biochemical activation. Thr 286 phosphorylation and cyclin D1 degradation were strongly induced by these treatments, but blocked by siRNA against ATR. Critically, siRNA against ATR also suppressed Thr 286 phosphorylation in normally cycling cells, as we had postulated. The conclusion that ATR is able to directly or indirectly promote cyclin D1 phosphorylation was then extended when dsDNA breakage was similarly shown to induce cyclin D1 phosphorylation through the related checkpoint kinase ATM. ATM is critical in cell cycle checkpoint blockage following DNA damage, raising the possibility that cyclin D1 might actually play a role in the DNA damage induced checkpoint response. To test this possibility cells were injected with a plasmid expressing at near endogenous levels the cyclin D1 T286A mutant, which cannot be phosphorylated and would therefore remain stable. dsDNA damage normally arrests cell cycle progression during G1 phase with high efficiency in these p53-containing cells. Of those cells expressing stable cyclin D1, however, almost 50% were induced to enter S phase; indicating that cyclin D1 suppression is vital to the DNA damage induced checkpoint response. This connection between DNA damage response kinases and cyclin D1, which normally serves to ensure genome stability, must be circumvented in formation of cancers as the result of genomic alterations.

425/B372

The Anti-Apoptotic E3 Ubiquitin Ligase Bre1p Protects from DNA Replication Stress in S.cerevisiae.

D. Walter, B. Fahrenkrog; M.E. Müller Institute for Structural Biology, Biozentrum, Basel, Switzerland

Inhibitor-of-apoptosis proteins (IAPs) are able to inhibit caspases due to the presence of two zinc-binding motifs, i.e. baculovirus IAP repeat (BIR) and RING domains. RING domains can recruit E2-ubiquitin conjugating enzymes and catalyze the transfer of ubiquitin onto target proteins. In search for RING domain containing proteins that might be implicated in yeast apoptosis, we show here that the E3 ubiquitin ligase Bre1p is able to protect against apoptosis. Bre1p is known to be required for the ubiquitylation of histone H2B and only recently *Δbre1* mutants were found to be sensitive to ionizing radiation, supporting a putative implication of Bre1p in yeast apoptosis. Moreover, C.elegans

BRE1 was identified in a screen for anti-apoptotic proteins. We found that yeast cells lacking *bre1* are more sensitive to apoptosis induced by hydrogen peroxide (H_2O_2), whereas overexpression of Bre1p confers resistance to H_2O_2. Moreover, chronologically aged cells lacking *bre1* show an earlier onset of cell death. Importantly, Bre1p requires its E3 ubiquitin ligase activity to protect against apoptosis. Further, we propose that Bre1p is activating the G1/S checkpoint since *Δbre1* cells fail to arrest in G1 during chronological aging, whereas cells expressing enhanced levels of Bre1p arrest in G1 and show decreased mutation frequency as compared to wild type cells. While the anti-apoptotic properties of Bre1p seem to be independent of its role in the ubiquitylation of histone H2B, DNA replication stress response proteins and checkpoint kinases are likely targets for Bre1p action in apoptosis inhibition.

426/B373

Changes in Cell Proliferation after Spinal Cord Injury Differ throughout the CNS.

M. J. Carmichael[1,2], N. L. Hayes[2], R. S. Nowakowski[1,2]; [1]Biomedical Engineering, Joint Graduate Program of Rutgers University and UMDNJ-Graduate School of Biomedical Sciences, Piscataway, NJ, [2]Neuroscience & Cell Biology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ

It is well known that there is a proliferative response within the spinal cord after spinal cord injury (SCI). We sought to elucidate whether SCI elicits a similar response within centers of the CNS distant from the lesion. Utilizing a single injection of bromodeoxyuridine (BrdU) thirty minutes before sacrifice to label cells within S-phase, we characterized the changes in cell proliferation within the CNS of adult, male C57BL/6J mice 96 hrs after SCI. We found changes in the number of BrdU labeled cells within regions of the CNS distant from the site of injury, namely the brain stem and dentate gyrus (DG). Within the brain stem, we found an 80% increase in S-phase cells ($p < 0.05$) predominately in white matter tracts, suggesting that this proliferative response after SCI is glial in nature. Conversely, there is a 20 - 30% decrease ($p < 0.01$) in the number of S-phase cells within the DG after SCI. Additionally, we assessed locomotor ability one day after injury and found that the magnitude of the decrease in cell proliferation within the DG correlates with injury severity. Taken together, our results indicate that acutely after SCI: (1) there are changes in cell proliferation in regions of the CNS distant from the lesion, and (2) these changes differ quantitatively and qualitatively in the areas studied. These results provide insight into potential targets of acute intervention after SCI that may be important for understanding and predicting response to treatment(s), rehabilitation and successful recovery after injury.

427/B374

Geminin Regulates Blood Cell Proliferation.

K. M. Shinnick, E. A. Eklund, T. McGarry; Medicine, Northwestern University Feinberg School of Medicine, Chicago, IL

During the cell cycle genomic integrity is maintained by checkpoint and feedback mechanisms. Bypassing these safeguards can lead to genetic instability and malignant transformation. The unstable protein Geminin limits the extent of DNA replication to one round per cell cycle by binding and inhibiting the essential replication factor Cdt1. Geminin is destroyed at the metaphase/anaphase transition by ubiquitin-dependent proteolysis, which allows a new round of replication in the next cell cycle. Over-expression of Cdt1 accelerates the development of lymphomas in $p53^{-/-}$ mice, but it is not known if Geminin acts as a tumor suppressor under physiological conditions. To examine this question, we created a mouse strain in which Geminin is deleted from the proliferating cells of the bone marrow. We found that Geminin deletion has profound effects on all three hematopoietic cell lineages. The production of mature erythrocytes and leukocytes is drastically diminished, and a population of immature leukocytes appears. In contrast, the population of polyploid megakaryocytes is dramatically expanded and the animals develop thrombocytosis. Interestingly, the population of c-Kit$^+$ Sca1$^+$ Lin$^-$ (KSL) stem cells is maintained at least in the short term. We conclude that Geminin is required for the production of all types mature blood cells except megakaryocytes. We are testing whether Geminin deficient cells give rise to leukemias or lymphomas after transplantation.

428/B375

Elg1p, an Alternative Replication Factor C Complex, Functions in Sister Chromatid Cohesion.

M. Maradeo, R. Skibbens; Biological Sciences, Lehigh University, Bethlehem, PA

Replication factor C (RFC) complexes catalyze the loading of PCNA-like sliding clamp complexes onto primed DNA. RFC complexes consist of four small subunits plus one of the four interchangeable large subunits. Rfc1-RFC is the only essential RFC complex and is utilized for processive DNA replication. Ctf18-RFC and Rad24-RFC function in various DNA repair processes. Elg1-RFC functions in homologous recombination, replication fork restart, S phase checkpoint pathways and Okazaki fragment maturation (Bellaoui et al 2003, Ben-Aroya et al 2003, Kanellis et al 2003, Banerjee and Myung 2004, Ayora and Kupiec 2005). Of the four RFC complexes, only Ctf18-RFC has been identified as promoting sister chromatid pairing - or cohesion (Skibbens et al 1999, Mayer et al 2001, Hanna et al 2001). However, physical interaction between all RFC subunits and the cohesion establishment factor Ctf7p/ Eco1p suggests that additional RFC complexes may be involved in chromatid cohesion (Kenna and Skibbens 2003, Satish and Skibbens, unpublished data). Here, we demonstrate a new pathway for the Elg1-RFC complex in chromatid cohesion. In contrast to the synthetic lethal effect ctf18 has on ctf7 mutants, deletion of elg1 rescues both temperature sensitivity of ctf7^eco1-1 and cohesion loss. While elg1 rescues ctf7^eco1-1 phenotypes, elg1 and mcd1-1 are synthetically sick. This evidence suggests that Elg1p and Ctf18p play opposing roles in cohesion establishment.

429/B376

The Lectin ArtinM Activates RBL-2H3 Cells without Inducing Degranulation.

P. A. Buranello, M. C. Roque-Barreira, M. C. Jamur, C. Oliver; Cell and Molecular Biology, FMRP USP, Ribeirão Preto, Brazil

Mast cells are immunoregulatory cells that participate in inflammatory and allergic reactions such as asthma, parasite elimination, angiogenesis and tissue regeneration. In order to perform these functions, mast cell activation is necessary. The plant lectin ArtinM (formerly designated as KM+) activates various cell types of the innate immune system by binding to mannose containing molecules on the cell surface, thus accelerating the process of wound healing and epithelial tissue regeneration. The ability of ArtinM to activate mast cells and stimulate their proliferation was investigated using the mast cell line, RBL-2H3. Cells were cultured in media with (0.25µg/ml) or without ArtinM. The relative growth of the RBL-2H3 cells using either crystal violet or alamar blue dyes showed that 0.25µg/ml of ArtinM increased

cell proliferation. After 24h more cells (34%) cultured with Artin M entered into mitosis when compared with controls (27%). ArtinM also accelerated the cell cycle as determined by FACS analysis after propidium iodide staining. This result was confirmed by BrdU incorporation. Anti-phospho-histone H3 was used to evaluate entry into the early stages of mitosis. In cultures maintained in media with ArtinM, there was a greater percentage of cells with phosphorylated histone H3. Incubation with 0.25μg/ml ArtinM was able to stimulate proliferation of RBL-2H3 cells without inducing liberation of β-hexosaminindase. These results were the same whether or not the cells were sensitized with IgE. Therefore, the ability of ArtinM in low concentrations to stimulate proliferation without stimulating degranulation, may contribute to the ability of ArtinM to promote tissue repair.

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CRL4-Cdt2 E3 Ubiquitin Ligase Co-operates with PCNA to Promote the Degradation of the Cell-Cycle Inhibitor p21.

T. Abbas, K. Terai, A. Dutta; Biochemistry, University of Virginia, Charlottesville, VA

Cul4 and Cdt2, components of the CRL4-Cdt2 E3 ligase are over-expressed in breast and prostate cancers and glioblastomas and the overexpression is correlated with advanced disease. We are screening substrates of CRL4-Cdt2 whose degradation will promote the genomic instability characteristic of advanced cancer. One substrate of CRL4-Cdt2 E3 ubiquitin ligase complex, the replication initiation factor Cdt1, has been shown to require interaction with PCNA for polyubiquitination and degradation. We now report a second substrate of CRL4-Cdt2, the CDK inhibitor p21, which is also known to associate with PCNA. Human cells that are depleted of components of CRL4-Cdt2 are deficient in the ubiquitylation and degradation of p21 after radiation. CRL4-Cdt2 also promotes the degradation of p21 during an unperturbed S phase of the cell cycle. The physical binding of p21 with PCNA is necessary for the efficient ubiquitylation of p21 by CRL4-Cdt2. Cdt2 functions to recruit the substrate p21 to the ubiquitin ligase complex. The CRL4-Cdt2 E3 ubiquitin ligase ubiquitylates p21 both in vivo and in vitro and its activity is dependent on the interaction of p21 with PCNA. Since accumulation of p21 after DNA damage is critical for the G1-S checkpoint that allows cells to repair the damage before DNA replication, our findings suggest that the overexpression of Cul4 or Cdt2 seen in late stages of many cancers will interfere with this checkpoint function and promote the fixation of mutations from the DNA damage. In addition, the results suggest the CRL4-Cdt2 E3 ligase has a special role in the degradation of proteins associated with the DNA polymerase processivity factor, PCNA, and so could modulate the levels of other proteins involved in maintenance of genome stability.

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Polo-like Kinase-2 Regulates Centriole Duplication through Degradation of the SCF-type Ubiquitin-Ligase Fbw7/hCdc4 Leading to CyclinE Stabilization.

I. Hoffmann, O. Cizmecioglu; DKFZ, Heidelberg, Germany

In mammalian cells, the centrosome consists of a pair of centrioles and amorphous pericentriolar material. The centrosome duplicates once per cell cycle. Polo like kinases (Plks) perform crucial functions in cell-cycle progression and during mitosis. The polo-like kinase-2, Plk2, is activated near the G1/S phase transition, and plays an important role in the reproduction of centrosomes. We show that Plk2 collaborates with cyclinE/Cdk2 in regulating centriole duplication although no direct interaction between these proteins could be detected. Our studies further indicate that Plk2 regulates cyclinE protein stability since both RNAi-mediated down-regulation of Plk2 or expression of a kinase-inactive Plk2 lead to a decrease of cyclinE protein levels. This results in a diminution of phosphorylation levels of centrosomal cyclinE/Cdk2 substrates, namely NPM/B23 and CP110. Interestingly, we find that Plk2 RNAi leads to stabilization of Fbw7/hCdc4, an SCF-type protein-ubiquitin ligase involved in the ubiquitin-mediated proteolysis of cyclinE. Plk2 directly phosphorylates and interacts with Fbw7/hCdc4 both in vivo and in vitro. Phosphorylation of Fbw7/hCdc4 by Plk2 occurs on three sites, S25, S176 and S349, leading to proteasome-mediated degradation of Fbw7/hCdc4, cyclinE stabilization and centriole duplication. Mutation of the three phosphorylation sites to alanine block degradation of Fbw7/hCdc4. Our results demonstrate that Plk2 and its substrate Fbw7/hCdc4 are critical components of the pathway underlying centriole reproduction.

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Deletion of the G2 Checkpoint Pathway Downstream Mediator of BRCA1, Wee1, in the Mammary Gland Results in Miscoordination of the Cell Cycle and Extensive DNA Damage.

A. Vasilopoulos, Y. Tominaga, R. Wang, C. Deng; NIDDK/NIH, Bethesda, MD

Previous studies have shown that Wee1 mediates the cell cycle arrest upon DNA damage induced by BRCA1 by inhibitory phosphorylation of Cdc2. Although the role of BRCA1 has extensively studied, the exact role of Wee1 in adult tissues has not yet been elucidated. Thus, the objective of our study was to unravel the effect of Wee1 deletion specifically in the mammary gland. Therefore, a Cre-loxP system was used as a method to disrupt the kinase activity of Wee1 by deleting exons 9 and 10, where Cre is under the control of the promoter of the gene encoding mouse mammary tumor virus-long terminal repeat (MMTV). Both whole-mount preparations and hematoxylin/eosin staining showed increased ductal branching in mammary glands of 6 months old female mice lacking Wee1. Since Wee1 is implicated in G2/M checkpoint, we examined the cell cycle progression. Our data revealed that Wee1 deficient mammary glands displayed higher levels of BrdU-stained cells compared to wild type in the absence of apoptosis and this could account for the enhanced ductal branching. When sections were stained for both phosphorylated histone H3 (phospho-H3), and Aurora B, common mitotic markers, higher number of cells from the mutated mammary glands showed the characteristic large punctate foci at the periphery of the nucleus, implying that these cells are cycling through mitosis. Interestingly, majority of these positive phospho-H3 stained cells displayed BrdU incorporation within the same nucleus. In contrast no cells were costained for BrdU and phospho-p27, a marker of late G1. This observation indicates that Wee1 mutated cells prematurely enter mitosis prior to the completion of S-phase. Thus, next we examined whether these cells experience spontaneous endogenous DNA damage. Indirect immunofluorescence with antibodies against mouse 53BP1 and phospho-H2AX resulted in increased levels of DNA damage foci formation relative to wild-type tissues. In conclusion, these data suggest a model in which deficiency of Wee1 can result in failure to restrain mitotic entry in the presence of DNA damage in vivo, while the effect of this phenomenon to genomic instability and tumor formation remains to be determined.

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Live Cell Imaging of the Induction of Aberrant Microtubule Nucleation at the Perinuclear Region of Mitotically Arrested Cells Treated with Microtubule-destabilizing Agents.

S. Sakaushi[1], K. Senda-Murata[1], S. Oka[1,2], K. Sugimoto[1]; [1]Graduate School of Life and Environmental Science, Osaka Prefecture University, Sakai, Japan, [2]Research and Development Center, Nagase & Co., Ltd., Kobe, Japan

Microtubule-targeting drugs disturb mitotic spindle formation and function by suppressing the dynamics of spindle microtubules. However, little is known about the process of microtubule disorganization or aberrant spindle formation induced by these drugs. To analyze in detail the aberrant spindle formation process in living cells, we constructed a multicolor MDA-MB-435 cell line in which the microtubules, centrosomes/spindle poles and chromatin were simultaneously visualized by stably expressing mCherry-α-tubulin, EGFP-Aurora A and ECFP-histone H3. We captured time-lapse images of early mitotic cells that were arrested in the subsequent mitotic phases by microtubule-destabilizing agents, nocodazole and vinblastine, or by a microtubule-stabilizing agent, paclitaxel. These microtubule-targeting drugs disturbed early steps of spindle formation in different manners as nuclear envelope breakdown occurred; nocodazole and vinblastine induced multiple asters at the separated centrosomes as well as at the perinuclear region, while paclitaxel inhibited the centrosome separation and the aster formation. These observations indicate that disturbance of the microtubule nucleation/organization occurs concurrent with nuclear envelope breakdown and that the perinuclear region of prophase cells has a potency to nucleate the microtubules. The microtubule nucleation activity at the perinuclear region may be weak and masked in the normal state but tends to be unmasked when cells are arrested by microtubule-destabilizing agents but not when cells are arrested by microtubule-stabilizing agents.

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Regulation of Cdk1 Activity in Early Mitosis: A Novel Mechanism for Modulation of Kinase Signal Strength.

S. Harvey[1], G. Enciso[2], J. Gunawardena[2], D. Kellogg[1]; [1]Department of Molecular, Cell, and Developmental Biology, UC Santa Cruz, Santa Cruz, CA, [2]Department of Systems Biology, Harvard Medical School, Boston, MA

Cyclin-dependent kinase 1 (Cdk1) initiates cell division. The Wee1 kinase phosphorylates and inhibits Cdk1 to delay cell division until conditions are appropriate. Here, we show that Wee1 also functions in a mechanism that modulates levels of Cdk1 activity in early mitosis. Budding yeast Wee1 is phosphorylated by Cdk1, which stimulates Wee1 to phosphorylate and inhibit Cdk1. The PP2A-Cdc55 phosphatase opposes phosphorylation and activation of Wee1 by Cdk1, thereby creating a kinetic barrier that prevents activation of Wee1 at low levels of Cdk1 activity. The PP2A-Cdc55 barrier allows a low level of Cdk1 activity to escape Wee1 inhibition to initiate early mitotic events. These observations show that opposing kinase and phosphatase activities can be used to modulate signal strength.

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Functional Analysis of Kinesin Family Member C1 (KIFC1) during Cell Cycle in Mammalian Cells.

N. Kim; Biochemistry, Yonsei University, Seoul, South Korea

The kinesin superfamily proteins (KIFs) are ATP-dependent microtubule motor proteins. KIFs transport organelles, protein complexes and mRNAs to specific destinations along microtubules while hydrolyzing ATP for energy. They also participate in chromosomal and spindle movements during mitosis and meiosis. Most KIFs have plus-end directed motility. However, some KIFs have a catalytic core at the C-terminus and have reversed (minus-end directed) motility (C-type motors). The Kinesin family member C1 (KIFC1) belongs to the kinesin-14 family C-type motors. In our study, KIFC1 was isolated as a protein that interacts with the stathmin homology region of centriolin by a yeast two-hybrid screen using Human Fetal Brain cDNA library. We showed that KIFC1 directly interacts with stathmin (also known as Oncoprotein18/Op18) by co-immunoprecipitation assay in HeLa cells. Stathmin is known to regulate microtubule dynamics by depolymerize microtubules during mitotic entry. Several kinases phosphorylate stathmin to inactivate the activity after prophase. Like stathmin, we found that the KIFC1 was also localized at the centrosome and mitotic spindle during mitosis. Overexpression of the KIFC1 cells showed delayed G2/M progression. These results suggest that KIFC1 might function in mitosis along with stathmin in mitotic entry. We are now investigating its mechanism to control mitosis.

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LCMT-1 Is Necessary for Proper Mitotic Progression.

J. A. Lee, D. C. Pallas; Biochemistry, Emory University, Atlanta, GA

Protein Phosphatase 2A (PP2A), a multifunctional Serine/Threonine (Ser/Thr) phosphatase, has been implicated in the regulation of cell growth and proliferation, primarily at the G2/M transition, in the development of human cancers, and apoptosis. Because PP2A is involved in so many diverse processes, it is highly regulated by both non-covalent and covalent mechanisms that are still being defined. PP2A is methylated on its catalytic subunit C-terminal leucine alpha-carboxy group by Leucine Carboxy Methyltransferase-1 (LCMT-1) and is demethylated by Protein Methylesterase-1 (PME-1). Reversible methylation is the most specific cellular mechanism for regulating PP2A, and thus may have promise as a mechanism-based therapeutic target. Little is known about the molecular mechanisms and regulation of PP2A C subunit methylation by LCMT-1 that is specific to cell proliferation and cancerous cell growth. In this study we have analyzed the importance of leucine carboxyl methyltransferase-1 (LCMT-1) for PP2A methylation and cell function. We have evidence that downregulation of LCMT-1, and thus a reduction in PP2A methylation, results in cell cycle defects specifically during mitotis. We found that reduction of LCMT-1 protein levels results in up to a 70% reduction in PP2A methylation in HeLa cells, demonstrating that LCMT-1 is the major mammalian PP2A methyltransferase. In addition, LCMT-1 knockdown reduced the formation of PP2A heterotrimers containing the Balpha regulatory subunit and, in a subset of the cells, induced apoptosis, characterized by caspase activation, nuclear condensation/fragmentation, and membrane blebbing. Knockdown of the PP2A B-alpha regulatory subunit induced a similar amount of apoptosis, suggesting that LCMT-1 induces apoptosis in part by disrupting the formation of PP2A B-alphaAC heterotrimers. LCMT-1 knockdown cells and B-alpha knockdown cells were more sensitive to the spindle-targeting drug nocodazole, suggesting that LCMT-1 and Balpha are important for spindle checkpoint. Taken together, our results indicate that LCMT-1 is a key regulator of mitotic progression and thus PP2A methylation and B subunit association are important for proper cell cycle function.

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Calcium/calmodulin Binds to and Inhibits Aurora-A Kinase Activity at Anaphase Onset.

R. Ban, T. Urano; Biochemistry, Shimane University School of Medicine, Izumo, Japan

Aurora-A is frequently overexpressed in human tumors. It directly compromises the spindle checkpoint and abrogates p53 function, effectively bypassing two critical maneuvering systems that maintain genomic stability and protect cells from transformation. In this study, we show that calmodulin, a ubiquitous calcium-sensing protein, directly binds to the catalytic kinase domain of Aurora-A in a calcium-dependent manner. This binding interaction significantly inhibits the kinase activity of Aurora-A as well as TPX2 activated Aurora-A. Confocal laser microscopy shows that these two proteins colocalize at the spindle poles during early anaphase. Calcium ions regulate the metaphase-anaphase transition during mitosis. Thus our results establish calcium/calmodulin as a negative regulator of Aurora-A and suggest a functional coupling between calcium signaling and a mitotic kinase Aurora-A through calmodulin. Our findings also offer the potential for a novel approach for the design of drugs that target the inhibition of Aurora-A kinase activity in tumor cells.

438/B385

Coordinating Polarized Growth and Cell Division in Fission Yeast.

J. Moseley[1], M. Almonacid[2], A. Mayeux[2], A. Paoletti[2], P. Nurse[1]; [1]The Rockefeller University, New York, NY, [2]Institut Curie, Paris, France

The links between polarized cell growth and cell division are poorly understood. We demonstrate that the conserved cell polarity kinase Pom1, which localizes to cell ends, regulates a G2/M signaling network that exists at medial cortical nodes in fission yeast cells. Previous studies described the appearance of cytokinesis proteins in medial nodes during mitosis, and subsequent condensation of the nodes into the cytokinetic ring. Here, we show that these nodes exist throughout interphase, when they contain key mitotic regulators, the anillin-like protein Mid1, and multiple uncharacterized proteins. We describe the hierarchical assembly of node proteins into these structures, and through genetic epistasis experiments we show that node factors form a genetic network regulating entry into mitosis. The interphase nodes are positioned by positive spatial signals from the cell nucleus, and negative regulatory signals from growing cell ends and the cell polarity kinase Pom1. Based on quantitative localization of Pom1, we propose a model describing how spatial constraints on Pom1 regulation of interphase nodes can both generate size information and link polarized growth with mitotic entry. Tests of the model confirm that spatial regulation of Pom1 kinase activity controls G2/M signaling at the medial cortical nodes. This work provides a potential molecular link between cell polarity and CDK regulation at the G2/M transition.

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Sphingosine-1-Phosphate Lyase Enhances Apoptosis in Response to Genotoxic Drug and Radiation by Releasing the Cells from G2/M Arrest.

A. Kumar, B. Oskouian, J. Saba; Saba Lab, Children Hospital Oakland Research Institute, Oakland, CA

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid metabolite that promotes cell survival, inhibits apoptosis and is irreversibly degraded by the enzyme sphingosine-1-phosphate lyase (SPL). Overexpression of SPL enhances apoptosis in response to stressful stimuli including DNA damage, but the cellular basis of this effect is not fully understood. Exposure of SPL-expressing 293T (SPL-T) cells to radiation induced apoptosis in a dose-dependent manner. SPL-T cells showed enhanced DNA damage and a distinct cell cycle profile after exposure to radiation. Twelve hours after radiation, control and SPL-T cells were arrested in G2/M phase; but after 24-30 hours, SPL-T cells exited the checkpoint while control cells were still arrested in G2/M. Pretreatment of SPL-T cells with nocodazole or inhibitors of aurora kinases, p53 or PI3K corrected the cell cycle profile and rescued SPL-T cells after radiation, indicating that the enhanced cell death observed in SPL-T cells is due to mitotic catastrophe. To confirm the specificity of SPL-mediated effects, a gene knockdown approach was employed. Stable shRNA-mediated SPL knockdown corrected the cell cycle profile and abrogated etoposide- and radiation-induced cell death in SPL-T cells. To determine whether the sensitivity of SPL-T cells is attributable to depletion of cellular S1P, we evaluated whether overexpression of sphingosine kinase 1 (SK), the major enzyme responsible for S1P synthesis, could reverse the effect. Surprisingly, we found SK expression did not attenuate the sensitivity of SPL-T cells. Consistent with this finding, pretreatment of SPL-T cells with S1P further potentiated cell death in response to DNA damage. Interestingly, treatment of control 293T cells with hexadecanal, the product of the SPL reaction, induced DNA damage and promoted early exit from the cell cycle after radiation, resulting in a cell cycle profile similar to SPL-T cells. Based on these findings, we conclude that SPL potentiates apoptosis in response to DNA damage by mediating early release of cells from G2/M, thereby promoting mitotic catastrophe. Further, SPL potentiates DNA damage through a process that involves accumulation of hexadecenal and occurs independently of S1P signaling.

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Subcellular Localization of Cyclin Dependent Kinase 6 in Multiple Cell Types.

D. Kohrt, J. L. Crary, V. Gocheva, M. Grossel; Department of Biology, Connecticut College, New London, CT

Cyclin dependent kinase 6(cdk6), is known to regulate G1 phase of the cell cycle by partnering with D-type cyclins to phosphorylate pRb. Historically, the role of cdk6 has been understood to be redundant with that of cdk4, a homologous D-type cyclin. However, recent evidence from several labs using a variety of model systems has shown an emerging role for cdk6 in the process of differentiation. Importantly, this function is not shared with cdk4, exposing for the first time meaningful functional differences between these D-cyclin kinases. Data from our lab showed that expression of cdk6, but not cdk4, in primary mouse astrocytes resulted in drastic changes in cellular morphology that correlated with changes in expression patterns of known markers of glial differenatiation (Ericson et al. Molecular Cancer 2003). Overexpression of cdk6 in these cells influenced the differentiation status of these astrocytes toward a less-differentiated progenitor-like cell. While the mechanism of cdk6 function in this process is not understood, we have demonstrated cdk6-induced changes in cell motility and changes in actin dynamics associated with differentiation of mouse astrocytes. These data suggest a role for cdk6 in the cytoplasm. To begin to understand the mechanism of cdk6 function in these cellular processes we have used multiple approaches to carefully examine the sub-cellular localization of cdk6 in multiple cell types. Preliminary data of cell fractionation, indirect immunofluorescence and GFP-fusions all indicate a predominately cytoplasmic localization of cdk6 in these cells. These data are consistent with a role for cdk6 in the cytoplasm.

Apoptosis I (441-458)

441/B388

The Expression of Caspase-3-dependent Apoptosis in Mural Thrombi Leukocytes.

P. Chu; Medicine, Chang Gung Memorial Hospital and University, Taipei, Taiwan

Background—Failure of arteriovenous access is mostly due to graft thrombosis and multifactorial, with medical and surgical etiologies. Apoptosis of blood cells, such as macrophages, lymphocytes and eosinophils, may play an important role in thrombus formation. We also investigated caspase-3-dependent apoptosis in thrombi. Methods and Results—We recorded clinical parameters in 43 consecutive patients with vascular access failure (13 men, 30 women; mean age±SD, 64.6±14.2 years) who underwent surgical thrombectomy. Major presentations included absent (92%) and/or near near-absent (16%) flow through the access during hemodialysis. Cardiovascular risk factors were hypertension (70%), hyperlipidemia (47%), diabetes mellitus (47%), chronic obstructive pulmonary disease (12%), heart failure (12%), coronary artery disease (21%), and stroke (16%). Laboratory data included a hemoglobin level of 100±17 g/L, total white blood cell count of 7.65±2.14 × 109/L, and a platelet count 205.6±57.9 1000/µL. Abnormal biochemistry data included elevated blood urea nitrogen level of 63.5±24.4 mg/dL and a creatinine level of 8.6±4.0 mg/dL (normal, <1.4 mg/dL). Thrombi were characterized by apoptosis (32%) in a caspase-dependent pathway in all types of leukocytes. Conclusion—Thrombi in arteriovenous access failure demonstrate apoptosis by means of the caspase-3 pathway in white blood cells.

442/B389

Proteomic Analysis of Myelogenic Leukemia Cells Undergoing Apotosis Induced by Idarubicin Combined with Proteasome Inhibitor (PS-341, bortezomib).

A. P. Doeskeland[1,3], G. Gausdal[1], A. Staes[4], J. Vandekerckhove[4], N. L. Larsen[1], B. T. Gjertsen[2], S. Doeskeland[1]; [1]Biomedicine, Univ. of Bergen, Bergen, Norway, [2]Medicine, Univ. of Bergen, Bergen, Norway, [3]PROBE, Univ. of Bergen, Bergen, Norway, [4]Medical Prot. Res., Univ. of Ghent, Ghent, Belgium

The synergistic effect of combining anthracycline (Idarubicin, Ida) and proteasomal inhibitor PS-341 (Velcade®, bortezomib) in inducing leukemia cell death is still not understood. We compared the proteome of IPC-81 acute myelogenic cells treated for 6 h with 100 nM Ida (45% apoptosis), 30 nM PS-341 (15% apoptosis) alone or in combination (95% apoptosis). Control cells were prelabeled for 6 generations with [13C]arginine (SILAC labeling) and combined with nonlabeled ([12C]arginine) cells treated with apoptosis inducers. Internal N-terminal peptides (arising from apoptosis-associated protein cleavage) were chemically modified and enriched by diagonal HPLC. The identity and 12C/13C ratio of these neopeptides (and of ordinary N-terminal peptides) was determined by HPLC-MS/MS. In general, more than half of the apoptosis-associated cleavage was at sites not recognized by caspases. In addition, some proteins appeared to become completely degraded based on decrease of N-terminal peptides and Western blots. Subset 1 of proteins, many previously associated with apoptotic processing, were processed under all three apoptosis-inducing conditions. This similarity could be due to proteasomal inhibition by Ida, which has been reported to bind to the proteasome. Ida failed, however, to inhibit the chymotrypsin-like proteasome activity in the cells. Neither did Ida treatment alter the level of the mature forms of the proteasome β5 and/or β5i subunits mainly responsible for the chymotrypsin-like activity. Probably subset 1 represented proteins generally processed in apoptosis. Several proteins (Subset 2) were processed only in response to Ida or Ida + PS-341, and a few (subset 3) only with PS-341 or PS-341 + Ida. More importantly, some proteins (subset 4) were processed only when Ida and PS-341 were combined. These proteins - containing notably mitochondrial or ER-associated proteins - may provide leads for unraveling the mechanism behind the synergy between Ida and PS-341 in inducing apoptosis.

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Targeting IGF-I Pathways to Induce Colorectal Cancer Cell Apoptosis.

S. Yang, K. M. Sales, B. Fuller, A. M. Seifalian, M. C. Winslet; University Department of Surgery, University College London, London, United Kingdom

Current chemotherapy agents are not highly effective against disseminated colorectal cancers. One major contributor to the limited effectiveness of the treatment is cancer cell producing high level of IGF-I and IGF receptors which has been proven protecting a broad range of colon cancer cells from a variety of apoptotic challenges caused by chemotherapy agents. The IGF system, therefore, has become an attractive molecular target for anticancer therapies. Based on IGF-I structure we have designed and synthesised a novel IGF type I receptor antagonist. The effect of the antagonist on human colon cancer cell proliferation was examined by a non-radioactive assay; the apoptosis was revealed by determining the activities of cellular caspases 3/7 8, 9. The apoptosis pathways were investigated by examining the level of pro-apoptosis singling protein with Western blotting. The results showed that IGF-I receptor antagonist induce cancer cell apoptosis and inhibits colon cancer cell proliferation. The changes of Caspase 3/7, 8 and 9 activities and different expression level of Bax in cancer cells after treatment with the peptide suggested that the extrinsic pathway may play an important role in IGF-I receptor antagonist induced apoptosis in colon cancer cells. Treating different type of colorectal cancer cells by combination of IGF-I receptor antagonists with current chemotherapeutic agents has shown that IGF-I receptor antagonists significantly augment sensitivities of colorectal cancer cells to current chemotherapeutic agents. All these data suggested that IGF-I receptor antagonists could be developed to a therapeutic strategy for colorectal cancer. Key word: novel IGF-I receptor antagonist

444/B391

Neuronal Nitric Oxide Synthase Contributes to Myocardial Ischemic Injury Whilst Still Is Required for Cardioprotection.

A. Barua[1], N. B. Standen[2], M. Galinanes[1]; [1]Cardiovascular Sciences, University of Leicester, Leicester, United Kingdom, [2]Cell Physiology & Pharmacology, University of Leicester, Leicester, United Kingdom

Objectives: Endothelia nitric oxide synthase (eNOS) is protective against myocardial ischemic injury but the role of neuronal nitric oxide synthase (nNOS) remains unclear. Therefore the aims of these studies were to: (i) investigate the role of nNOS in ischemia/reoxygenation-

induced injury and (ii) determine whether it is species dependent. Materials and Methods: Myocardial slices (30-40mg) obtained from the right atrial appendage of patients undergoing elective cardiac surgery and from the ventricles of rat, mice, and nNOS knocked out mice, were subjected to 90min ischemia and 120min reoxygenation (37oC). Muscles were randomized for treatment with NO donor SNAP (100µM), non selective NOS inhibitor L-NAME (100µM) and selective nNOS inhibitor TRIM (100µM) for 20min prior to ischemia (n=6/group). Some preparations were subjected to ischemic preconditioning (IP: 5min ischemia, 5min preconditioning). Tissue injury was assessed by creatine kinase (CK) released into the media during the reoxygenation period (IU/mg wet wt), and cell necrosis and apoptosis was determined by propidium iodide and TUNEL (% of aerobic control). Results: L-NAME significantly reduced CK leakage (0.23±0.06 vs. 0.66±0.04 in control; $p<0.05$), necrosis (7.7±0.8 vs.13.1±0.5 in control; $p<0.05$) and apoptosis (6.5±1.1 vs. 22.1±1.9 in control; $p<0.05$) in rat myocardium. Greater reduction of injury was induced by TRIM (0.03±0.01, 2.8±0.5 and 3.5±0.8 respectively; $p<0.05$ vs. L- NAME) and by SNAP (0.03±0.01, 2.1±0.40 and 2.0± 0.36 p= NS vs. TRIM), an effect that was superior to IP (0.27±0.03, 7.0±0.7and 9.1±1.1 $p<0.05$ vs. TRIM). Similar results were seen with mice and human myocardium. In nNOS knockout mice IP could not be induced and TRIM and L-NAME did not have significant protective effect whereas SNAP almost abolished the injury. Conclusions: These results indicate that nNOS plays a dual role by causing ischemic injury and at the same time being necessary to achieve protection by IP, a role that is similar in all species studied.

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PKA-mediated Phosphorylation of 14-3-3 during Translation - Relation to cAMP-induced Leukemia Cell Apoptosis.

S. Doeskeland, R. Kleppe, E. Kjarland, K. Viste, S. Huseby, K. Broenstad; Biomedicine, Univ. of Bergen, Med. Fac., Bergen, Norway

Background: 14-3-3 proteins are chaperones binding to protein epitopes phosphorylated by Ser/Thr kinases. Of the 7 mammalian 14-3-3 proteins several can protect against apoptosis, e.g. by displacing Bcl-2 from phospho-Bad, increasing the level of free Bcl-2. We have shown previously that IPC-81 leukemia cells become committed to apoptosis after a 2 hour pulse of increased cAMP. At this time they have a stable, high level of phospho-Bad, an increased level of the pro-apoptotic Bcl2 family member Bim, and can no more be rescued from apoptosis by translational inhibitors. Findings: 1) Using 2D-PAGE to detect cAMP-induced protein spots in the IPC cells, we identified an acidic shift of 14-3-3g by MS/MS, verified by antibody against 14-3-3 phosphorylated on Ser 59. 2) A steady increase of P-14-3-3g was noted during the critical 2 hours until the cells became committed to apoptosis. 3) The IPC cell 14-3-3 phosphorylation depended completely on translation and active cAMP-dependent kinase (PKA). 4) This was reproduced in vitro, PKA phosphorylating Ser59 of 14-3-3g only during active translation. 5) FPLC analysis showed that PSer59-14-3-3g was unable to dimerize, but readily heterodimerized with 14-3-3e. 6) Biacore analysis showed that PSer59-14-3-3 was unable to bind phospho-Bad, and that its hetereodimer with 14-3-3e bound very weakly. Conclusions: a) This is the first demonstration of 14-3-3 protein phosphorylation on Ser 59 in intact cells. b) The cotranslational nature of the phosphorylation is rare, and suggests that the consequences are most marked at sites of active translation, like close to the mitochondrion where the Bcl-2 family members act in apoptosis. c) A probable scenario is that phosphorylation of juxta-mitochondrially synthesized 14-3-3 promoted the cAMP- induced apoptosis by blocking the ability of non-modified 14-3-3 protein to neutralize phosho-Bad, thereby lowering the threshold for apoptosis induction by Bim.

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Oncogenic Ras Induces Autophagic Cell Death through MKK7/JNK-mediated Upregulation of Atg5.

J. Byun, M. Kim, C. Yoon, S. Lee; Hanyang University, Seoul, South Korea

To prevent the development of malignancies, mammalian cells activate disposal programs, such as programmed cell death, in response to deregulated oncogene expression. However, the molecular basis for regulation of cellular disposal machinery in response to activated oncogenes is unclear at present. In this study, we show that oncogenic H-Ras (H-rasV12) induces autophagic vacuole formation and caspase-independent cell death in normal fibroblasts. Moreover, MKK7/JNK/c-Jun-mediated upregulation of the autophagy-related protein, Atg5, is required for H-rasV12-induced cell death. We observed marked autophagic vacuole formation and cell death in normal fibroblasts overexpressing H-rasV12, which remained unaffected by a caspase inhibitor. However, pretreatment with Bafilomycin A1, an autophagy inhibitor, completely attenuated H-rasV12-induced caspase-independent cell death and autophagic vacuole formation. Selective Atg5 production was observed in cells overexpressing H-rasV12, and siRNA targeting Atg5 clearly inhibited autophagic vacuole formation and cell death. Interestingly, JNK- or c-Jun-specific siRNA, but not ERK- or p38 MAPK-specific siRNA, suppressed Atg5 upregulation, autophagic vacuole formation, and caspase-independent cell death. The JNK-activating kinase, MKK7, was selectively activated in response to H-rasV12, and MKK7 inhibition effectively attenuated JNK activation and autophagic vacuole formation. These data support the theory that activation of the MKK7/JNK/c-Jun signaling pathway promotes upregulation of Atg5 in response to oncogenic Ras, resulting in autophagy and subsequent caspase-independent cell death. Our findings suggest that in cells acquiring apoptotic defects, oncogenic stress triggers autophagy, which protects against malignant progression.

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Location of the Myocardial Protective Effect Exerted by Exogenous NO and the Inhibition of nNOS.

A. Barua[1], N. B. Standen[2], M. Galinanes[1]; [1]Cardiovascular Sciences, University of Leicester, Leicester, United Kingdom, [2]Cell Physiology and Pharmacology, University of Leicester, Leicester, United Kingdom

Objectives: Nitric oxide (NO) is cardioprotective and a mediator of ischemic preconditioning (IP). We have shown that the activity of neuronal nitric oxide synthase (nNOS) is detrimental during ischemia but at the same time is necessary for cardioprotection by IP. However, the location of these effects in the transduction pathway remains unclear. The aim of this study was to determine the location of the beneficial effect exerted by exogenous NO and the relationship of nNOS with mitoKATP channels and p38MAPK, two key components of IP transduction pathway. Materials and Methods: Myocardial slices (30-40mg) obtained from rat ventricles were subjected to 90min ischemia followed by 120min reoxygenation at 37oC. Muscles were randomised for treatment with NO donor SNAP (100µM), selective nNOS inhibitor TRIM (100µM), mitoKATP channel blocker 5HD (1mM) and p38MAPK inhibitor SB203580 (10µM) for 20 min prior to ischemia (n=6/group). Some preparations were subjected to ischemic preconditioning (IP: 5min ischemia, 5min preconditioning). Tissue injury was

assessed by creatine kinase (CK) released into the media during the reoxygenation period (IU/mg wet wt), and cell necrosis and apoptosis was determined by propidium iodide and TUNEL (% of aerobic control). Results: Both SNAP and TRIM significantly decreased CK release (0.15±0.03 and 0.23±0.02), cell necrosis (4.0±0.6 and 4.6±0.7), and apoptosis (2.8±0.6 and 3.3±0.3) when compared to control (0.79±0.05, 17.3±3.2 and 15.1±1.7 respectively; $p<0.05$ in all cases). The protective effect induced by SNAP and TRIM was blocked by SB203580 (0.60±0.07, 12.7±0.9 and 10.4±1.3 for SNAP; 0.76±0.09, 13.6±1.4 and 11.7±0.8 for TRIM; p=NS vs. control) but not by 5HD (0.19±0.02, 3.7±1.0 and 4.3±1.3 for SNAP; 0.25±0.04, 5.0±1.1 and 4.7±0.9 for TRIM; $p<0.05$ vs. control). As expected, both SB203580 and 5HD blocked protection by IP. Conclusion: These results show that the protection obtained by exogenous NO and by inhibition of endogenous NO produced by nNOS are exerted upstream of p38MAPK and downstream of mitoKATP channels.

448/B395

Cell Cycle Arrest and Apoptosis Induced by a Novel G-Quadruplex Stabilizer.

Y. Tsai[1], H. Qi[1], C. Lin[1], E. J. LaVoie[2], J. E. Rice[2], D. S. Pilch[2], L. F. Liu[1]; [1]Pharmacology, UMDNJ-RWJMS, Piscataway, NJ, [2]Pharmaceutical Chemistry, Rutgers, The State University of New Jersey, Piscataway, NJ

A synthetic hexaoxazole-containing macrocycle, HXDV, has been shown to exhibit specific binding to human telomeric G-quadruplexes in vitro. The molecular mechanism of HXDV-induced M phase arrest of cell cycle and apoptosis signal was studies. HXDV inhibits the growth of tumor cells and induces robust apoptosis within 12 hours independent of the telomerase status, and telomere dysfunction was not observed. DNA content analysis has indicated that HXDV arrests cells at G2/M phase of cell cycle, while additional studies have suggested that HXDV induces M phase arrest rather than G2 phase arrest of cell cycle: (1) HXDV-arrested cells exhibit condensed chromosomes and separated centrosomes, (2) Mitotic index increases about 5 fold in cells treated with HXDV, (3) HXDV, unlike DNA damaging agents, induces dephosphorylation rather than phosphorylation of Cdc2 at Tyr-15, indicative of cell entry into mitosis, (4) HXDV stimulated histone H3 phosphorylation at Ser-10, another mitosis marker, and (5) Studies using time-lapse microscopy have also shown that HXDV-treated cells prolong the process of chromosome condensation, spindle fiber assembly and nuclear membrane breakdown. One scenario explaining the abnormal chromosome dynamics is a defect in the microtubule-kinetochore interaction in mitosis. The centromeric protein A (CENP-A) is a key component of kinetochore inner plate proteins, and many function of CENP-A are dependent on its phosphorylation by Aurora kinases. Further comparison between the cellular effect of microtubule targeting drugs and HXDV, shows that HXDV can inhibit the CENP-A phosphorylation level in a dose-dependent manner while microtubule targeting drugs, either polymerization stabilizer (taxol) or destabilizer (vinblastin), increase the signals at the same phosphorylation site, suggesting both classes of chemicals do not share similar mechanism accounting for the M phase arrest of cell cycle and apoptosis signals. Our results suggest that HXDV can perturb the chromosome dynamics, resulting in M phase arrest of cell cycle and apoptosis activation through a mechanism involving the regulation of centromere function. This study also suggests a role of G-quadruplex conformation in centromere biology.

449/B396

A Novel Role for Ajuba LIM Proteins in Organ Size Control in Drosophila Epithelia.

M. Das Thakur[1,2], M. J. Seppa[4], J. B. Skeath[3], G. D. Longmore[1,2]; [1]Medicine, Washington University in St. Louis, St. Louis, MO, [2]Cell Biology, Washington University in St. Louis, St. Louis, MO, [3]Genetics, Washington University in St. Louis, St. Louis, MO, [4]Developmental Biology, Washington University in St. Louis, St. Louis, MO

The determination of organ size during development requires an understanding of the biological processes that control cell size and number within an organ. In general these include coordinated regulation of cell proliferation and cell death decisions through cell intrinsic regulatory pathways or in response to environmental cues (i.e., extrinsic regulation). Importantly, the disruption of these regulatory mechanisms is a hallmark of cancer. We have identified the Drosophila ortholog of the mammalian Ajuba LIM protein family to be necessary for normal organ growth during Drosophila development. The expression of 2 different RNAi constructs targeting dAjuba (dCG11063) as well as dAjuba gene deletion through FLP-FRT recombination using Exelixis transposon insertions all result in the same phenotypes: (1) ubiquitous expression results in late embryonic to early larval lethality; (2) when targeted to the developing wing small adult wings result due to a decrease in cell number and increased apoptotic cell death; (3) when driven in the pupal eye there is a loss of interommatidial epithelial cells also due to increased apoptosis. Recent studies in Drosophila and mammals have implicated the Hippo/Salvador/Warts/Yorkie pathway as an important cell intrinsic regulator of organ size and cancer development. Loss of function of Hippo/Salv/Warts result in increased cell numbers due to reduced cell death caused by unchecked Yorkie-mediated upregulation of cyclin E and Drosophila inhibitor of apoptosis (DIAP1) levels. When dAjuba loss-of-function flies were screened for genetic interaction with the Hippo pathway we found a strong genetic interaction with Yorkie. These genetic studies coupled with biochemical interactions between Ajuba family LIM proteins and members of the Hippo pathway implicate Ajuba LIM proteins as negative regulators of the Hippo - Yorkie pathway regulating organ cell numbers.

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Constraints on the Structure of Ceramide to Achieve Targeted Channel Formation.

M. Perera[1], V. Ganesan[1], L. Siskind[2,1], Z. Szulc[2], A. Bielawska[2], R. Bittman[3], M. Colombini[1]; [1]Department of Biology, University of Maryland, College Park, MD, [2]Medical University of South Carolina, Charleston, SC, [3]City University of New York, Queens College, New York City, NY

The sphingolipid, ceramide, self-assembles in phospholipid membranes, forming large channels capable of translocating proteins across said membranes. These channels are believed responsible for protein release from mitochondria, a key, decision-making step in apoptosis. Chemical analogs, with alterations in each of the major structural features of ceramide, were examined for their ability to form channels in phospholipid membranes and mitochondrial outer membranes. The results demonstrate the over-riding importance of a hydrogen-bonded network in stabilizing the structure of the channel. Alternative and mutually-incompatible channel structures were observed as well as synergistic behavior between ceramide and specific analogues. These all point to a highly-organized channel structure, similar to those formed by proteins. Remarkably, the ability of ceramide to form channels in mitochondrial membranes and not plasma membranes is very sensitive to any modification, indicating a strong evolutionary selection pressure for this distinction, enforced by sensitive molecular

recognition. As a further indication of the tight evolutionary window in which ceramide operates, increasing the flexibility of the hydrocarbon chains results in inhibition of cytochrome oxidase. Thus the structural constraints on ceramide are necessary to allow it to form large channels specifically in mitochondria and avoid affecting other cellular processes. (supported by NSF grant: MCB-0641208 and a Nano-Biotechnology DBED award)

451/B398

Autophagy Protects Yeast from Sulforaphane-induced Cell Death.

B. Roussel, N. Austriaco; Providence College, Providence, RI

Sulforaphane (SFN) is a member of a class of antioxidants known as isothiocyanates that are found in broccoli and other cruciferous vegetables. Work from several laboratories has shown that SFN has anticancer and antimicrobial activity though its mechanism of action has not yet been clearly elucidated. Several studies in mammalian cells have suggested that SFN may act by inducing programmed cell death (PCD). To further elucidate the mechanism of action of SFN, we have initiated studies of its effects on the budding yeast, Saccharomyces cerevisiae. We have determined that SFN kills wild type yeast cells. Moreover, we have discovered that sulforaphane induces autophagy and that cells unable to undergo autophagy because of a null mutation in the key autophagic gene, ATG1, manifest a heightened sensitivity to the drug. Finally, we have data suggesting that wildtype cells chemically induced to undergo autophagy show greater resistance to SFN treatment than cells that were not induced suggesting that autophagy may be a protective mechanism induced in cells to protect themselves against SFN-mediated cell death.

452/B399

An All-or-None Switch for Apoptosis Is Linked to a Threshold Level of BAX.

S. Semaan[1,2], R. Nickells[2]; [1]Biomolecular Chemistry, University of Wisconsin Madison, Madison, WI, [2]Ophthalmology and Visual Sciences, University of WI Madison, Madison, WI

The pro-apoptotic gene *Bax* is required for death of several neuronal populations, including retinal ganglion cells (RGCs). Recently, we identified two mouse lines where mice heterozygous for a mutant *Bax* gene exhibit two different RGC death phenotypes after optic nerve crush. DBA/2J$^{Bax+/-}$ RGCs survive, similar to *Bax* deficient mice, whereas 129B6$^{Bax+/-}$ RGCs die, similar to mice wildtype for *Bax*. Quantitative PCR revealed twice the level of *Bax* mRNA in 129B6 neurons compared to DBA/2J neurons. These results suggested that a threshold level of *Bax* was required for cell death. We tested this idea in vitro in the HCT116 human colon cancer cell line that are dependent on *BAX* for death induced by the non-steroidal anti-inflammatory drug, indomethacin. A GFP-BAX fusion construct was cloned downstream of a Tetracycline-responsive promoter utilizing pTRE-Tight. HCT116$^{BAX-/-}$ cells were transiently co-transfected with pTET-On and pTRE-Tight containing GFP-BAX or containing GFP. The levels of GFP-BAX or GFP were titrated using increasing concentrations of doxycycline (DOX) in media. After 24 hours, cells were treated with indomethacin to induce the apoptotic program and cell death was assayed 48 hours later. From 0.1 ng/ml to 0.5 ng/ml of DOX, no difference in cell death was observed in cells transfected with GFP-BAX compared to cells transfected with GFP alone. Each condition yielded 8-10% cell death. However, at the concentrations of 1 ng/ml to 1 µg/ml DOX, cells transfected with GFP-BAX exhibited equivalent levels of cell death in the range of 30-35% ($P = 0.47$). In comparison, cells transfected with GFP at the same DOX concentrations exhibited only 8-12% cell death, which is a significant difference in cell death (GFP-BAX vs. GFP $P = 6.1X10^{-6}$). This study indicates that the level of BAX in a cell must exist at a certain threshold level in order for cell death to occur in response to an apoptotic stimulus. At this threshold, cell death in response to the amount of BAX can be best described as an all-or-none effect. Due to a threshold effect, in *Bax*$^{+/-}$ mice from different backgrounds, the small difference in the levels of *Bax* quite possibly determines whether an RGC dies or survives.

453/B400

Echis carinatus Venom Induction of Apoptosis in HEK 293 Cells.

S. Kim, R. Pierce, C. Borgerding, E. Albrecht; Biology & Physics, Kennesaw State University, Kennesaw, GA

Echis carinatus produces potent hemorrhagic venom that causes inflammatory disorders including the development of apoptotic and necrotic tissues. In this study, we investigate the relationship between polyethyleneimine (PEI) substrate concentration and Echis carinatus venom induced apoptosis. HEK 293 cells were grown for 24 hours on tissue culture plates coated with 0.25, 2.5, or 25 µg/ml polyethyleneimine (PEI), then stimulated for 3 or 12 hours with 100 µg/ml Echis carinatus venom or 10 µM staurosporine (positive control). HEK 293 cells grown on 0.25 µg/ml PEI, then stimulated for 3 hours with Echis carinatus venom displayed a significant reduction in cell viability, compared to cells grown on 2.5 or 25 µg/ml PEI. Increasing stimulation to 12 hours caused a reduction of cell viability at each substrate concentration. However, cells grown on 2.5 and 25 µg/ml PEI were significantly protected against cell death compare to cells grown on 0.25 µg/ml PEI. In contrast, cells stimulated for 12 hour with staurosporine showed significant reduction in viability at each substrate concentration. Using an adherence assay, we found cells grown on 0.25 µg/ml PEI, had a 55.0±11.8 percent reduction in adherence after 3 hours of Echis carinatus stimulation. This suggests Echis carinatus venom may reduce cell viability indirectly by eliminating their contact with the substratum, thereby inducing a contact dependent form of apoptosis, termed anoikis.

454/B401

Characterization of the Survival Effect Induced by Hypoxia-induced Gene-1a (Hig-1a) of RAW264.7 in Hypoxic Stress.

H. Shin; Biology, Chung nam National Univ, Dae jeon, South Korea

Hig-1a is a novel gene that can be induced in the condition of hypoxia, deprivation of glucose, and serum starvation. It is a 10.6 kDa and mitochondrial inner membrane protein, which is oriented with N' outside-C' outside and loop inside. There are 3 different types of Hig found in mouse, Hig-1a, Hig-1b and Hig-2a. We found Hig-1a was the most significantly induced by hypoxia and Hig-1b was hardly detectable. Recently, over-expression of human Hig-1a was reported to induce survival effect against stimuli of hypoxia and low glucose in pancreatic beta cells. Therefore we undertook experiments to investigate whether Hig-1a is a generalized survival factor against cell death stress. First, we cloned 300 bp upstream region of Hig-1a that has a strong transcriptional activity for Hig-1a to test transcriptional regulation. It has two

HRE (hypoxia response element) sites. Among them, HRE (-35~-31) is the responsible element for the transcriptional activity. Mutation of the site could abolish the transcription of Hig-1a induced by hypoxia, low glucose and nitric oxide. Second, stably transfected RAW264.7 macrophages with Hig-1a were produced. The Hig-1a cells showed significantly reduced apoptosis compared with control cells in hypoxic condition. Third, when endogenous Hig-1a was removed by transfection with antisense cDNA of Hig-1a, the hypoxia induced apoptosis was strongly enhanced. Forth, the survival effect induced by Hig-1a was related with reduced activities of Caspase-3 and 9. Lastly, Hig-1a deleted with N-terminal 26 a.a. completely abolished the survival effect demonstrating that the N-terminal region residing in the interspace of mitochondria is inevitable for the survival effect.

455/B402

A General Anti-apoptotic Pathway Mediated by Activating Transcription Factor 5 in Human Solid Tumors.

Z. Sheng[1,2], M. R. Green[1,2]; [1]Howard Hughes Medical Institute, Worcester, MA, [2]PGFE, UMASS Med Sch, Worcester, MA

Activating transcription factor 5 (ATF5) is inhibited at transcription level after cytokine or growth factors withdrawal. It is highly expressed in tumors and plays an essential role in antagonizing apoptosis. However, the molecular mechanisms that drive ATF5 expression and its antagonized apoptosis remain to be elucidated. Here we exploited a novel strategy in genome-wide shRNA library screening using diphtheria toxin. We identified 12 shRNAs suppressing endogenous ATF5 transcription in GL261 mouse glioma cells, three of which (Frs2->Pak1->Creb3l2) functioned sequentially. Our further analyses demonstrated that a signaling cascade through FGFR/EGFR/Frs2 to Ras/Pak1/MAPKs governs both Creb3l2 and ATF5 expression and Creb3l2 directly regulates ATF5. Given the fact that interfering ATF5 causes apoptosis in glioma cells, we further analyzed the ATF5 regulatory pathway and apoptosis. Our results showed that inhibitors or shRNAs which attenuate ATF5 regulatory pathway triggered apoptosis which was then, at least partially, blocked by ectopically expressed FLAG tagged ATF5. Similar to sorafenib which significantly represses Mcl1, we found that ATF5 regulates Mcl1. To study the generality of this pathway, we then analyzed 6 human glioblastoma cell lines and 10 solid tumor cell lines generated from different tissues and found that those cells were sensitive to sorafenib which suppressed both ATF5 and Mcl1. Ectopically expressed ATF5 antagonized sorafenib induced apoptosis in those cells and more importantly ATF5 siRNA induced apoptosis and concomitantly decreased Mcl1. Our results indicated that a signaling cascade regulates ATF5 and its downstream anti-apoptotic pathway which is general in human solid tumors.

456/B403

Protein Profiling of Diepoxybutane-treated Human Lymphoblasts.

E. Martinez-Ceballos; Biological Sciences and Environmental Toxicology, Southern University, Baton Rouge, LA

Butadiene (BD) is a known mutagen and human carcinogen prevalent in petrochemical industrial areas. Diepoxybutane (DEB) is considered to be the most active metabolite responsible of the observed effects of BD in humans. In immortalized human lymphocytes, DEB induces apoptosis through the activation of the p53 tumor suppressor. Although we and others have found that p53-deficient human lymphocytes exhibit an increased resistance to the apoptotic effect of DEB, little is known about the mechanism of DEB action in p53-deficient cells. In the present work, we have employed Sigma's Cell Signaling Panorama© Antibody Arrays to examine the protein expression levels of NH32 (p53-deficient) versus TK6 (p53-proficient) human lymphoblasts with or without DEB treatment (5μM) for 48 hours. Changes in protein expression patterns after DEB treatment were similar for both NH32 and TK6 cells, with the exception of the cytoskeleton components Cytokeratin, Fibronectin, and MAP1, which were upregulated by DEB treatment (> 2-fold) in NH32 cells but not in TK6 cells. Conversely, the signal transduction proteins JNK, MAPK, PKCα, and PKCγ, which play pro-apoptotic roles in some cell types, were upregulated by DEB (> 2-fold) in TK6 cells but not in NH32 cells. The results obtained from the antibody array experiments were validated by Western blot and immunofluorescence analyses. Because upregulation and re-arrangement of cytoskeleton components is a strategy followed by diverse cell types to escape apoptosis, our results provide an insight into the molecular mechanism by which p53-deficient lymphoblasts are able to resist the effects of DEB in culture.

457/B404

Mitochondria Shuttling of CAP1 Promotes Actin- and Cofilin-dependent Apoptosis.

C. Wang[1], G. Zhou[1], S. Vedantam[1], P. Li[2], J. Field[1]; [1]Pharmacology, University of Pennsylvania, Philadelphia, PA, [2]Biology, Hong Kong University of Science and Technology, Clear Water, China

Mitochondria play a central role in regulating apoptosis by releasing proapoptotic contents such as cytochrome c, and generating reactive oxygen species (ROS). Early in apoptosis, proteins translocate to mitochondria to promote the release of their contents. Here, we show that the actin and cofilin interacting protein, CAP1 (adenylyl Cyclase-Associated Protein), has a role in apoptosis. When we induced apoptosis, CAP1 rapidly translocated to the mitochondria independent of caspase activation. Translocation was induced by Staurosporine and Etoposide treatment, and was observed in multiple cell lines including HeLa, NIH3T3, HEK239 and COS-7. CAP appears to translocate to the outer membrane of mitochondria. Translocation was pro-apoptotic because CAP1 knockdown cells were more resistant to apoptosis inducers. Overexpression of wild-type CAP1 did not stimulate apoptosis on its own but stimulated cofilin-induced apoptosis. Apoptosis induction required a mitochondrial-targeting domain, localized in the N-terminus and additionally the actin binding domain in the C-terminus. Taken together these studies suggest that CAP1 provides a direct link from the actin cytoskeleton to the mitochondria by functioning as an actin shuttle.

458/B405

Mitochondrial Outer Membrane Proteins Facilitate Bax-mediated Lipidic Pore Formation in the Absence of Cardiolipin.

B. Schafer[1], J. Quispe[2], R. Schneiter[3], T. Kuwana[1]; [1]Department of Pathology, University of Iowa Carver College of Medicine, Iowa City, IA, [2]National Resource for Automated Molecular Microscopy, The Scripps Research Institute, La Jolla, CA, [3]Department of Biochemistry, University of Fribourg, Fribourg, Switzerland

Mitochondrial outer membrane permeabilization (MOMP) is a critical step in apoptosis and is regulated by Bcl-2 family proteins. In vitro systems based on artificial cardiolipin-containing liposomes can reproduce the key features of MOMP; however, the nature of the 'pores'

remains controversial. Examining permeabilization in cardiolipin-liposomes by cryo-electron microscopy that allows us to visualize the membranes with minimal processing, we discovered large round holes with diameters of 25-100 nm suggestive of lipidic pores. We addressed another puzzling issue: whereas liposomes required ~7 % cardiolipin, outer membranes underwent permeabilization with much less of that lipid. We found that detergent-solubilized MOM components could be reformed into vesicles that responded to Bcl-2 family proteins despite the absence of detectable levels of cardiolipin. Furthermore, we isolated MOM proteins from the outer membrane vesicles by extracting and removing the lipids using organic solvent and found that the incorporation of isolated MOM proteins into liposomes without cardiolipin conferred responsiveness to Bcl-2 family proteins. Taken together, we conclude that MOM proteins promote Bax-mediated lipidic pore formation in the absence of cardiolipin.

Growth Factors and Receptors (459-480)

459/B406

Adipocytokines Mediate Growth and Differentiation of Human Pulpal Cells.

I. Tamura, A. Kamada, S. Goda, Y. Yoshikawa, E. Domae, T. Ikeo; Biochemistry, Osaka Dental University, HIrakata, Japan

Adipocytokines, such as adiponectin and leptin, are expressed by adipocytes and mediate fatty acid oxidation in fat tissue as well as glucose concentration in serum. Recently, they are also thought to mediate calcification in mineralized tissue. We investigated the influence of adiponectin or leptin on the kinetics of human pulpal cells using ELISA and Western blotting techniques. All experiments were approved by the Ethics Committee of Osaka Dental University (No. 050351). We obtained human pulpal cells from three patients upon gaining informed consent and have maintained under Dulbecco's modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). After cultivation in DMEM without FBS for two days, pulpal cells from the 7th to 9th passages were incubated with DMEM with 100 ng/mL of adiponectin or 10 ng/mL of leptin for 1, 2, 3 or 4 days, respectively. We confirmed that human pulpal cells express adiponectin receptor 1 and leptin receptor, but not adiponectin receptor 2. From this time course experiment, the proliferation index of these cells as measured by 5-bromo-2'-deoxyuridine incorporation increased in the presence of either adiponectin or leptin. Alkaline phosphatase activity showed a tendency to increase after stimulation with adiponectin and not to significantly change after stimulation with leptin. Adiponectin induced the expressions of bone morphogenetic protein (BMP)-2, dentin sialoprotein and runt-related transcription factor (Runx) 2 over a time course in contrast to gradually reduced expression of osteopontin. Pulpal cells stimulated with leptin increased the expressions of dentin sialoprotein and osteopontin, but the leptin stimulation showed no correlation with the expression of BMP-2 and Runx2. These results indicate that adiponectin may play a role in dentin calcification by influencing the growth and differentiation of human pulpal cells, while leptin may influence the formation of dentin extracellular matrices.

460/B407

A Hepatocyte Growth Factor Antagonist Engineered by Site-directed Mutagenesis of HGF/NK1.

F. Cecchi[1], D. Pajalunga[2], C. Fowler[2], B. Peruzzi[1], N. MacDonald[2], D. k. Grella[2], P. T. Wingfield[3], S. J. Stahl[3], J. D. Kaufman[3], A. R. Byrd[4], D. P. Bottaro[1]; [1]Urologic Oncology Branch, NIH/NCI, Bethesda, MD, [2]EntreMed, Inc., Rockville, MD, [3]Protein Expression Laboratory, NIH/NIAMS, Bethesda, MD, [4]Structural Biophysics Laboratory, NIH/NCI, Frederick, MD

Hepatocyte growth factor (HGF) is a heparan sulfate (HS) binding protein that stimulates survival, proliferation, motility and morphogenesis in a variety of target cells expressing the receptor tyrosine kinase Met and HS glycans. HGF/NK1 is a naturally occurring truncated HGF isoform consisting of the amino-terminal (N) and first kringle (K1) domains, and stimulates all major HGF-associated biological activities. Within NK1, the N domain binds HS, while K1 contains Met binding residues. To further define the role of HS in receptor activation and signaling, we engineered opposite charge mutations at selected putative HS binding residues in the N domain of NK1. The mutations did not disrupt the native NK1 structure, and Met binding was unchanged while HS binding was reduced relative to wild type (WT) NK1, most profoundly in the triple mutant K60E/K62E/R73E (3M). Motogenic and mitogenic activities were substantially diminished in specific mutants, and 3M was devoid of intrinsic activity and competitively antagonized HGF-induced DNA synthesis. Expression plasmids encoding 3M and WT were transfected into the glioblastoma cell line U87 MG, which is dependent on HGF/Met autocrine signaling for tumorigenicity in mice. 3M/U87 MG cells displayed a lower proliferation rate in culture relative to WT/U87 MG or the parental cell line, suggestive of HGF antagonism by 3M protein. In mice, expression of WT significantly accelerated the growth of U87 MG tumor xenografts, which reached maximal volume 22 days post-implantation, compared to 45 days required by the parental cell line. In contrast, tumor growth in animals injected with 3M/U87 MG was just measurable at 50 days post implantation. HGF and Met expression levels in each cell line were comparable, suggesting that the different growth rates were not due to the inadvertent selection of subpopulations with altered HGF/Met pathway activation levels. Thus HS binding is critical for HGF-driven motogenesis, mitogenesis and tumor growth, and inhibition of these activities by 3M validates a novel strategy for developing selective, potent antagonists of the HGF pathway and potentially those of other HS-binding ligands.

461/B408

Analysis of SUMO-mediated Stress Response in Saccharomyces cerevisiae.

K. Ro, A. Chattopadhyay, J. Wohlschlegel; Biological Chemistry, UCLA, Los Angeles, CA

Cells are constantly exposed to a wide variety of environmental stresses and their ability to maintain the integrity of their genome in the midst of these insults is essential to their survival. One cellular pathway that plays a key role in this cellular response is the SUMO modification pathway. SUMO is a member of the ubiquitin family of small protein modifiers and its covalent attachment to cellular targets is a critical regulatory step for a diverse range of cellular processes that includes transcriptional regulation, cell cycle progression, and DNA damage response. In this study, we have begun to investigate the role of the SUMO pathway in responding to ethanol- induced oxidative stress. Analysis using Western blots and spot assays reveal that ethanol stress increases SUMOylation and affects cell growth. Utilizing shotgun proteomics, we have identified several candidate SUMO substrates whose conjugation to SUMO is increased after ethanol treatment.

Specifically, 119 SUMO substrates were found to be present in greater abundance in ethanol treated cells versus untreated cells. We are currently characterizing the functional role of this set of SUMO targets in the cellular stress response. Ultimately, this work will enable us to understand how cells use the SUMO pathway to protect themselves from genotoxic damage and offer insight into how the deregulation of this pathway, a common feature in cancer, may lead to genetic instability.

462/B409

Regulation of Human Fibroblast Growth Factor Receptor 1 Expression by Transcription Factor E2F-1.

M. Kanai, E. Tashiro, M. Imoto; Keio University, Yokohama, Japan

[Objective] Overexpression of fibroblast growth factor receptors (FGFRs) has been observed in many types of human cancers. However, it is still largely uncharacterized about the regulatory mechanism of human FGFR expression. Therefore, it would be an important to elucidate the regulatory mechanism of FGFR expression for studying the role of FGFRs in tumor progression. Recently, we have shown that the transcription of mouse FGFR-1 and -2 were regulated by the pRB/E2F-1 pathway which was activated by overexpression of cyclin D1. Although we found that the FGFR-1 promoter sequence showed 70.8 % homology between mouse and human, the regulatory mechanism of human FGFR-1 (hFGFR-1) has not yet been clarified. Therefore, in this study, we examined whether the expression of human FGFR-1 (hFGFR-1) was also regulated by the pRB/E2F-1 pathway. [Results] Since the transcriptional initiation site of hFGFR-1 gene has not yet been characterized, we first determined it by 5'-RACE using total RNA from EC17 cells. Next, to examine whether the transcription of hFGFR-1 was regulated by E2F-1, we constructed hFGFR-1 promoter-luciferase plasmid containing -887/+60 region of hFGFR-1 gene relative to the transcriptional initiation site and we showed the luciferase activity of hFGFR-1 promoter-luciferase construct was increased in response to transient transfection of E2F-1. Furthermore, the analysis of various deletion mutants of hFGFR-1 promoter-luciferase construct and EMSA indicated that a sequence located within +4/+22 region is an E2F-1-responsive sequence. Finally, we examined whether hFGFR-1 protein expression was regulated by pRB/E2F-1 pathway in situ. The hFGFR-1 protein expression was up-regulated in LoVo cells infected with adenoviral E2F-1, but down-regulated in HT1080 cells infected with adenoviral pRB. [Conclusions] We demonstrated here that the expression of human FGFR-1 is regulated by the pRB/E2F-1 pathway.

463/B410

Nuclear Proinsulin C-peptide Stimulates Ribosomal Gene Expression.

E. Lindahl[1], U. Nyman[1], F. Zaman[1], J. Shafqat[1], T. Masaharu[2], L. Sävendahl[1], H. Jörnvall[1], B. Joseph[1]; [1]Karolinska Institutet, Stockholm, Sweden, [2]Okayama University Graduate School of Medicine, Okayama, Japan

C-peptide, the connecting segment of proinsulin, is released from pancreatic beta cells in amounts equimolar with those of insulin. Thus, type I diabetic patients have a deficiency of C-peptide in addition to that of insulin, and several malfunctions in type I diabetic patients as well as in streptozotocin diabetic rats are alleviated by C-peptide via partly unknown mechanisms. We have previously shown that C-peptide is internalized into cells (Lindahl E., et al. 2007), but a function of its intracellular localization has not been established. By using an array of cell biology tools, including confocal microscopy, RT-PCR and a novel mass spectrometry cross-linking technique, we show that C-peptide upon cellular entry localizes to the nucleus where it promotes transcription of ribosomal RNA genes and increase histone acetylation within hours. This represents a novel mechanism by peptide hormones to rapidly induce RNA transcription, and implies that C-peptide has growth factor activity.

464/B411

Identification of Usp18 as a Regulator of EGF Receptor Synthesis.

J. E. Duex, A. Sorkin; Department of Pharmacology, University of Colorado Denver, Aurora, CO

Abnormally high levels of epidermal growth factor receptor (EGFR) contribute significantly to the progression of many types of cancer. However, the cellular mechanisms which control EGFR levels, and therefore tumorigenic potential of cells, remain poorly understood. To identify proteins in cancer cells which are involved in regulating the levels of EGFR we developed a high throughput screen in squamous cell carcinoma cells. Screening was performed using a library of small-interfering RNAs which target various ubiquitination-related genes. This approach revealed a number of proteins whose depletion resulted in a significant reduction in the number of EGFRs at the cell surface. One of these genes, Usp18, is a member of the ubiquitin specific protease family. siRNA knock-down of Usp18 in several cell lines reduced expression levels of EGFR by 50-80%, while the levels of other receptor tyrosine kinases remained unchanged. Analyzing the synthesis and turnover of metabolically radiolabeled EGFR demonstrated that the rate of receptor synthesis was reduced up to 4 fold with the depletion of Usp18. Interestingly, the dramatic reduction in EGFR synthesis occurred despite EGFR mRNA levels remaining unchanged, suggesting that depletion of Usp18 affected the translation step of EGFR synthesis. Moreover, the effect of Usp18 depletion on EGFR mRNA translation required the presence of EGFR 5' and 3' untranslated region sequences. This observation suggests that Usp18 controls EGFR expression via regulatory elements within EGFR mRNA. Taken together our data provide evidence for a novel mechanism of EGFR regulation that involves control of EGFR synthesis by a ubiquitin protease.

465/B412

Bone Morphogenetic Protein 2 Mediated Interaction between Peroxisome Proliferator-activated Receptor γ and β-catenin Suggests a Novel Regulatory Mechanism in Endothelial Cell Survival.

T. Alastalo, V. de Jesus Perez, T. Kleisli, M. Rabinovitch; Pediatrics, Stanford University, Stanford, CA

Introduction: Our previous studies indicate that peroxisome proliferator-activated receptor γ (PPARγ) mediates the bone morphogenetic protein receptor II (BMPRII) signaling that is responsible for the anti-proliferative response in pulmonary arterial smooth muscle cells (PASMCs). In contrast BMPRII signaling has a pro-survival and pro-proliferative response in pulmonary artery endothelial cells (PAECs), which is dependent on the recruitment of Wingless/beta-catenin (Wnt/βC). Objective: We therefore hypothesized that PPARγ plays a role in the BMPRII mediated survival response in PAECs via interaction with βC. Methods: Human pulmonary microvascular and large vessel PAECs were compared. PPARγ agonist, Rosiglitazone (1μM), and antagonist, GW9662 (1μM), were applied to evaluate the role of PPARγ

in BMP mediated survival in serum-deprived conditions as determined by cell counts. Interaction and co-localization of PPARγ with βC were assessed by co-immunoprecipitation and confocal microscopy, respectively. Results:. PPARγ can physically interact with βC in PAECs assessed by immunoprecipitation and by confocal microscopy. Furthermore, this interaction is increased 2 fold upon BMP2 stimulation and coincides with the activation of βC. However one hour pre-treatment with both PPARγ ligands (Rosiglitazone and GW9662) inhibit BMP2 mediated PPARγ - βC interaction 45% and 35%, respectively. Consistent with this, Rosiglitazone and GW9662 inhibit BMP2-mediated large and microvessel PAEC survival by 50% and 30%, respectively. These results are supported by immunocytochemistry showing attenuation of BMP2-induced co-localization of PPARγ and βC in PAEC nuclei, with both PPARγ ligands. Conclusions: BMP2 mediated interaction between PPARγ and βC suggests a novel mechanism of cross-talk between BMP and Wnt/βC signaling. Increased complex-formation coincides with PAEC survival, while disruption with PPARγ ligands is associated with an attenuated survival response. We suggest that some synthetic PPARγ ligands can have detrimental effects on PAECs by affecting cell survival mechanisms that depend on the Wnt/βC signaling.

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A Metabolic and Energetic Role for Erythropoietin in Support of Cell Proliferation through Mitochondrial Biogenesis Regulators PGC-1alpha and AMPK.

M. Pham, B. Holmes, S. K. Edwards, T. Tsao; University of Arizona, Tucson, AZ

Proliferating cells have unique metabolic requirements beyond those of quiescent cells. Data in literature indicate that cell proliferation rate is coupled to cellular energetics. However, the mechanisms by which proliferating cells anticipate and meet sufficient metabolic requirements remain unclear. We observed in cytokine-dependent Ba/F3 cell line expressing the receptor for erythropoietin (Epo) a decrease in Epo-stimulated proliferation upon inhibition of mitochondrial oxidative phosphorylation by rotenone or dinitrophenol. Surprisingly, inhibition of oxidative phosphorylation and cell proliferation unmasked a function for Epo in stimulating lactate production. To further assess the role of mitochondria in cell proliferation and the metabolic functions of Epo, we examined the levels of oxidative phosphorylation markers and signaling molecules important for mitochondrial biogenesis in Ba/F3 cells expressing Epo receptor. Protein levels of cytochrome oxidase subunit IV (COXIV) and Complex III core protein 2 decreased precipitously following 24 hours of cytokine withdrawal. Interestingly, inhibition of Janus Kinase 2 (JAK2), the tyrosine kinase associated with Epo receptor, by AG490 elicited a similar decrease in COXIV even in the presence of Epo. In addition, Epo increased the levels of the mitochondrial biogenesis regulators peroxisome proliferator-activated receptor-gamma coactivator-1alpha (PGC-1alpha) and AMP-activated protein kinase alpha (AMPKalpha) in a JAK2-dependent manner in Ba/F3 cells. Both total and phosphorylated (activated) AMPKalpha were increased following Epo stimulation. Treatment with the AMPK inhibitor Compound C decreased Epo stimulation of COXIV, indicating a linear signaling cascade from JAK2 to mitochondrial biogenesis through AMPKalpha. In summary, Epo stimulates mitochondrial protein content by increasing the levels of mitochondrial biogenesis regulators PGC-1alpha and AMPKalpha. In addition to the well characterized signaling cascades that directly impact cell proliferation or survival, our data suggest a novel role for Epo in directing cellular metabolism and energetics through regulators of mitochondrial biogenesis.

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Neuropilin-1 Promotes PDGF Signaling and Pericyte Motility.

S. Cao[1], U. Yaqoob[1], R. Huebert[1], D. Mukhopadhyay[2], V. Shah[1]; [1]GI research unit, Mayo Clinic, Rochester, MN, [2]Department of Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN

PDGF-dependent pericyte recruitment is an essential step in angiogenesis and vascular remodeling, however, mechanisms which regulate PDGF signaling remain incompletely defined. Results: Focused microarray and real-time PCR analyses revealed that the axonal guidance molecule, neuropilin-1 (NRP-1) is upregulated in pericytes which have undergone activation into a highly motile myofibroblastic phenotype, and that NRP-1 colocalizes with the PDGF-receptor in a cellular and sub-cellular context. Complementary gain- and loss-of-function approaches revealed a dominant role for NRP-1 in the process of cell motility and chemotaxis in response to PDGF ligand. Mechanistically, NRP-1 increases PDGF binding affinity with its receptor thereby promoting receptor autophosphorylation and migration signaling. In summary, these results delineate a novel observation whereby NRP regulates PDGF dependent pericyte motility, and define mechanisms by which this effect is conferred. It is anticipated that these processes will have broad implications for pericyte-based angiogenesis and vascular remodeling.

468/B415

Nuclear Epidermal Growth Factor Receptor (EGFR) Interacts with Signal Transducer and Activator of Transcription 5 (STAT5) in Activating Aurora-A Gene Expression.

L. Hung; [1]Department of Pharmacology, Tainan, Taiwan, [2]Institute of Biosignal Transduction, Tainan, Taiwan

Loss of the maintenance of genetic material is a critical step leading to tumorigenesis. It was reported that overexpression of Aurora-A and the constitutive activation of the epidermal growth factor (EGF) receptor (EGFR) are implicated in chromosome instability. In this study, we examined that when cells treated with EGF result in centrosome amplification and microtubule disorder, which are critical for genetic instability. Interestingly, the expression of Aurora-A was also increased by EGF stimulus. An immunofluorescence assay indicated that EGF can induce the nuclear translocation of EGFR. Chromatin immunoprecipitation (ChIP) and re-ChIP assays showed significant EGF-induced recruitment of nuclear EGFR and signal transducer and activator of transcription 5 (STAT5) to the Aurora-A promoter. A co-immunoprecipitation assay further demonstrated that EGF induces nuclear interaction between EGFR and STAT5. A small interfering (si)RNA knockdown assay also showed that EGFR and STAT5 are indeed involved in EGF-increased Aurora-A gene expression. Altogether, this study proposes that the nuclear EGFR associates with STAT5 to bind and increase Aurora-A gene expression, which ultimately may lead to chromosome instability and tumorigenesis. The results also provide a novel linkage between the EGFR signaling pathway and overexpression of Aurora-A in tumorigenesis and chromosome instability.

469/B416

WDR3 Is Induced by IGF-I and Is Required for Ribosome Biogenesis and Cell Proliferation.

M. McMahon, R. O Connor; Biochemistry, University College Cork, Cork, Ireland

The Insulin-like Growth-Factor-I Receptor (IGF-IR) promotes cell growth, survival, and differentiation. Increased expression of genes downstream of an activated IGF-IR is thought to play an important role in mediating the growth and invasiveness of tumours. In order to identify IGF-I regulated genes associated with tumour growth and cancer progression we isolated a series of genes that were differentially expressed in the R- cell line (mouse embryonic fibroblasts derived from IGF-IR) that cannot be transformed compared with R+ cells (R- cells over expressing the IGF-IR) that are highly transformed. From this screen a series of genes associated with ribosome assembly and function were isolated. One such gene, WDR3 was found to be expressed higher in R+ cells compared with R- cells. WDR3 has being reported to be localised to chromosome 1p12-p13, a region of chromosome 1 which has being associated with malignancies. To date, the function of WDR3 has not previously being studied, however studies in yeast suggest it may play an important role in ribosome biogenesis. Therefore, in order to access how IGF-I signalling regulates ribosome function we sought to investigate the role of WDR3 in ribosome biogenesis and cell proliferation in mammalian cells. In this study WDR3 mRNA was found to be up-regulated by IGF-I in a number of human and murine cell lines, and the IGF-I mediated induction of WDR3 was found to require both the PI3-Kinase and mTOR signalling pathways. Analysis of RNA profiles and 32P in vivo RNA labelling assays revealed that siRNA mediated suppression of WDR3 resulted in altered ribosomal RNA processing. Using siRNA to suppress expression, we determined that WDR3 is required for proliferation of tumour cell lines. Cell cycle analysis revealed that suppression of WDR3 resulted in an accumulation of cells in the G1 phase of the cell cycle, and an up-regulation of a number of known tumour suppressor proteins which control entry in the S phase of the cell cycle. Overall the data suggest that WDR3 is a novel IGF-I regulated protein required for ribosome biogenesis and cell cycle progression in mammalian cells. Increased expression of WDR3 in tumour cells would enhance cancer progression.

470/B417

Lamellipodin Regulates Cell Proliferation in Response to EGF via SRF and Actin Dynamics.

A. Vehlow[1], M. Michael[1], E. Lyulcheva[2], E. Taylor[2,3], D. Bennett[2,3], M. Krause[1]; [1]Randall Division of Cell and Molecular Biophysics, King's College London, London, United Kingdom, [2]Department of Zoology, Oxford University, Oxford, United Kingdom, [3]School of Biological Sciences, University of Liverpool, Liverpool, United Kingdom

Lamellipodin (Lpd) belongs to the MRL protein family (Mig10, RIAM and Lpd), which link activated Ras GTPases with actin regulatory Ena/VASP proteins to induce local changes in cytoskeletal dynamics and cell motility. However, the upstream pathways regulating these changes are still unknown. We report here that Lpd is required for EGF-mediated cell proliferation. Indeed, stable knockdown of Lpd in HeLa cells results in reduced cell proliferation rates in response to EGF as measured by an MTT proliferation assay. We demonstrate that this phenotype is not due to increased apoptosis or cell death using FACS analysis of Annexin-V/7AAD staining. Lpd and RIAM alter cellular monomeric(G):filamentous(F) actin ratios, but the role of these changes has not been fully appreciated. We furthermore show that Lpd has the ability to activate serum response factor (SRF), a transcription factor that responds to changes in G:F-actin ratios via its co-factor Mal. Based on these findings we propose a role for Lpd in EGF-induced cell proliferation through its regulation of SRF signaling.

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A Receptor Neuropilin-1 Mediates HGF-induced Muscle Satellite Cell Quiescence.

M. Yamada[1], S. Shiratsuchi[1], R. Tatsumi[1], W. Mizunoya[1], Y. Ikeuchi[1], R. E. Allen[2]; [1]Bioscience and Biotechnology, Kyushu University, Fukuoka, Japan, [2]Animal Sciences, University of Arizona, Tucson, AZ

Postnatal muscle growth and regeneration rely on molecular events responsible for activation and quiescence of satellite cells. Hepatocyte growth factor (HGF) triggers satellite cell activation entry into the cell cycle in response to mechanical perturbation, and their subsequent expression of myostatin (MSTN) signals a return to quiescence. Our recent *in vitro* data showed that HGF could induce satellite cell quiescence by stimulating MSTN expression, but the HGF concentration required is much higher than that required for the satellite cell activation. These findings suggest that the events stimulated by high concentrations of HGF may be mediated by a low affinity receptor for HGF and not by the high affinity c-met receptor. Here we explore the possibility that the receptor neuropilin-1 (Npn-1) is involved in the HGF-MSTN pathway that signals satellite cells to return to quiescence. Experiments were conducted with primary cultures of quiescent satellite cells, which were prepared from S.D. male rats. Activation was stimulated from 24 to 48 hr post-plating, in DMEM-10% horse serum by 2.5 ng/ml human recombinant HGF, a concentration shown to stimulate activation. Cultures were then incubated in the media containing 500 ng/ml HGF with or without 5 µg/ml anti-Npn-1 neutralizing antibody for the next 24 hr. The neutralizing treatment inhibited MSTN expression and prevented satellite cells from returning to quiescence to a level comparable to control culture that received 2.5 ng/ml HGF. Analysis of whole cell lysates of satellite cells by western blotting and RT-PCR demonstrated the presence of Npn-1 protein and mRNA at 48 hr post-plating, which is the time at which neutralizing antibody was added to cultures in this study. Finally, in order to investigate the potential interaction between Npn-1 and HGF, 72-hr satellite cell lysate from the culture receiving 500 ng/ml HGF was subjected to Npn-1-immunoprecipitaion analysis, which clearly demonstrated that a 60-kDa HGF band co-precipitated with Npn-1. Therefore, these results provide evidence that Npn-1 is a strong candidate for an unknown HGF-binding partner that could play a crucial role in the MSTN-dependent return of satellite cells to quiescence.

472/B419

Lactoferrin - a Novel Bone Growth Factor.

D. Naot, J. Cornish, A. Chhana, K. E. Callon, I. R. Reid; Medicine, University of Auckland, Auckland, New Zealand

Lactoferrin, an 80kDa iron-binding glycoprotein present in milk and other exocrine secretions in mammals, is a pleiotropic factor involved in the regulation of immune function, embryonic development and inflammation. We have previously shown that lactoferrin is anabolic to bone at physiological concentrations. In vitro, lactoferrin stimulates the proliferation, differentiation and survival of osteoblasts, as well as potently inhibiting osteoclastogenesis in bone marrow cultures. In vivo, local injection of lactoferrin results in substantial increases in bone formation

and bone area. The aim of the current study was to investigate the mechanism of action of lactoferrin in bone cells by measuring changes in gene expression in osteoblasts treated with lactoferrin. Cultures of the MC3T3-E1 osteoblastic cell line were harvested at different time points after treatment and gene expression was compared to untreated cells by real-time PCR, using custom-designed microfluidic cards. The cards were designed to measure the relative expression levels of 48 genes including bone-specific transcription factors, matrix and inflammatory proteins. Lactoferrin induced a rapid, dose-dependent increase in the transcription levels of interleukin-6 (IL-6), interleukin-11 (IL-11), the pro-inflammatory factor prostaglandin-endoperoxide synthase 2 (COX-2) and the transcription factor nuclear factor of activated T cells-1 (NFATc1). Subsequent real-time PCR experiments in primary human osteoblasts confirmed that IL-6, IL-11, COX-2 and NFATc1 transcription levels increase significantly and transiently within two hours of lactoferrin treatment. The role of COX-2 and NFATc1 activation in mediating the mitogenic effect of lactoferrin in osteoblasts was investigated using specific inhibitors. We found that the addition of either the COX inhibitor Indomethacin or the NFATc1 inhibitor Cyclosporin A blocked the increase in proliferation induced by lactoferrin in a dose-dependent manner. In conclusion, lactoferrin is proving to be a positive regulator of bone with a possible physiological role in bone growth and healing, and a better understanding of its mechanism of action could contribute to its development as a bone anabolic factor.

473/B420

'Rotation/twist' Model for the Activation of the EGF/ErbB Receptor Family.

I. Maruyama; Molecular Neuroscience Group, Okinawa Institute of Science and Technology, Okinawa, Japan

The epidermal growth factor (EGF)/ErbB receptor family consists of four members, and plays pivotal roles in the development of organisms ranging from worms to humans. Aberrant activation of the receptors is frequently implicated in a variety of human cancers. Ligand-induced dimerization is proposed as a molecular mechanism underlying the activation of all the growth factor receptor tyrosine kinases including the ErbB receptor family. Before ligand binding, however, it remains controversial whether the receptors have a monomeric or dimeric structure. In this conference, we will show that all the ErbB receptors have preformed, yet inactive, homo- and heterodimeric structures either on the cell surface or in the nucleus, using a bimolecular fluorescence complementation (BiFC) assay. The spontaneous dimerization occurs in endoplasmic reticulum (ER), where ligands are unlikely to be available, before reaching the cell surface. These results are consistent with previous observations by in vivo chemical crosslinking, cysteine disulfide formation, FRET and FCCS. Based on current and previous results, we will propose the 'rotation/twist model' for the activation of the ErbB receptor family.

474/B421

Quantifying the Competition between Growth Factors and Cell-Cell Contacts in Epithelial Cell Cycle Regulation.

J. Kim[1], A. Asthagiri[2]; [1]Division of Engineering and Applied Science, California Institute of Technology, Pasadena, CA, [2]Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA

The release of normal epithelial cells from growth constraints is a principal driving factor in oncogenesis. Contact-inhibition of proliferation is one such constraint, and the loss of contact-inhibition has been implicated in hyperproliferation of epithelial cells and tumor formation. While the importance of contact-inhibition is well established, the quantitative aspects of this key constraint are poorly understood. In particular, it is unclear how contact quantitatively competes with soluble growth factors stimulating cell cycle activity, to make a "net" decision. In this work, we demonstrate that a quantitative titration of epidermal growth factor (EGF) and cell-cell contact regulates cell cycle activity of epithelial cells. Our data indicate that contact-mediated suppression of cell proliferation is sensitive to growth factor concentration. More specifically, only below a critical threshold level of EGF, cadherin-mediated contacts undergo maturation and suppress cell proliferation by selectively inhibiting Erk, but not Akt, signaling. Phenotypically, their coupling imparts a spatial dynamics in epithelial population growth, which is characterized by the onset of transient spatial gradient in cell cycle activity below this threshold EGF. Furthermore, we demonstrate that this critical threshold amount of EGF is tunable. Using micropatterned surfaces to simulate highly maturated cell clusters, we show that contact-inhibition can be triggered at significantly higher doses of EGF. This quantitative, tunable competition between EGF and contact may have implications for our understanding of development and cancer and for biomedical applications such as tissue engineering.

475/B422

Establishment of Overexpression System of Multifunctional CCN Family Proteins for Structural Analysis.

J. Kato[1,2], H. Mori[1], T. Nakanishi[1]; [1]Department of Pharmacy, Shujitsu University, Okayama, Japan, [2]Department of Medicine, Hyogo College of Medicine, Nishinomiya, Japan

CCN family consists of six proteins, CCN1-6, containing four conserved domains, insulin-like growth factor binding protein, von Willebrand factor, thrombospondin, and C-terminal cysteine knot domains. The family proteins modulate angiogenesis, chondrogenesis, fibrosis, and wound healing. Some of the family proteins have the same function, but the others do not although the family proteins share the structurally similar modules. We have been trying to overexpress human CCN family proteins to understand structural diversity of the family proteins. Protein overexpression is the first step for structural analysis of proteins. But, it is sometimes hard to obtain a soluble form of proteins when overexpressed in *E.coli*. We report a system of overexpression of CCN family proteins, which utilizes *E.coli* strains that highly express chaperon proteins. Human CCN1-3 genes were amplified by PCR and cloned into pET32b or pGEX-2T plasmid to give pGEX-GST-CCN1, pET32-HisCCN2, and pGEX-GST-CCN3, which produce GST-CCN1 fusion protein, N-terminal histidine-tagged CCN2 protein and GST-CCN3 fusion protein, respectively. Chaperone-overexpressing plasmids were transformed into *E.coli* BL21(λDE3) and plasmids for CCN1-3 fusion protein expression were then introduced into their BL21(λDE3). Chaperone-overexpressing plasmids included pG-KJE8 (DnaKJ, GrpE, GroELS), pGro7 (GroELS), pKJE7 (DnaKJ, GrpE), pG-Tf2 (GroELS, Tig), and pTf16 (Tig). Solubility of the expressed proteins was assessed by SDS-PAG/immunoblotting after *E.coli* fractionation. CCN fusion proteins were almost insoluble when expressed in the original BL21(λDE3) strain at 37°C. Thirty to eighty percent of expressed HisCCN2 protein was soluble when expressed in chaperone-overexpressing BL21(λDE3) at low temperature. Solubility of the overexpressed HisCCN2 protein was dependent on a combination of chaperones overexpressed in *E.coli*. Co-overexpression of DnaKJ, GrpE and GroELS gave the highest solubility of expressed HisCCN2.

Soluble forms of CCN1-GST and CCN3-GST were also obtained in the same expression strategy. This overexpression system can be applicable to improvement in the solubility of other proteins expressed in *E.coli*.

476/B423

Transforming Growth Factor Beta (TGF-β) Regulates the Forkhead Box O (FOXO) Transcription Factors in a Cell-specific Manner.

D. Ings, N. Pittman, J. Dore; Memorial University of Newfoundland, St. John's, NF, Canada

The FOXO family of transcription factors regulate a large number of genes involved in such varied functions as cellular replication and apoptosis. These transcription factors are downstream of Phosphotidylinositol 3-Kinase (PI3K) and are negatively regulated by AKT through phosphorylation. The PI3K pathway is positively regulated by the tyrosine kinase receptor family, however it has also recently been shown that TGF-β stimulates PI3K in mesenchymal cell lines. TGF-β is known to differentially regulate growth in a cell type dependent manner. Specifically, it is growth stimulatory to fibroblasts and inhibitory to epithelial and hematopoietic cell types. Together, this suggests that TGF-β should also regulate the FOXO family. This study examines TGF-β regulation of endogenous levels of FOXO family members in the context of phenotypically normal cell lines. Both epithelial (Mv1Lu and NMuMG) and fibroblast cell lines (AKR-2B) were treated with TGF-β (1ng/ml) for up to four hours. Total cellular proteins were analysed by western blot for FOXO1 and FOXO4. Epithelial cells showed that TGF-β1 treatment caused a time dependant decrease in endogenous levels of both phosphorylated FOXO1 and FOXO4 in Mv1Lu and NMuMG cells respectively. Conversely, fibroblasts showed an increase in FOXO1 and FOXO4 phosphorylation over time. This increase was shown to be PI3K dependent since LY294002 treatment resulted in a decrease in TGF-β induced phosphorylation. These results demonstrate that TGF-β regulates FOXO1 and FOXO4 phosphorylation in a cell type specific manner. Since, FOXO transcription factors are known to affect downstream cell cycle regulators, these results further suggest that TGF-β regulates cell growth in a cell type specific manner in part through the FOXO family.

477/B424

Cell and Molecular Mechanisms of Insulin-induced Angiogenesis.

Y. Liu[1,2], M. Petreaca[1], M. Martins-Green[1]; [1]Cell Biology and Neuroscience, UC Riverside, Riverside, CA, [2]Burn Department, Ruijin Hospital, JiaoTong University Medical College, Shanghai, China

Angiogenesis, the development of new blood vessels from pre-existing vessels, is a key process in the formation of the granulation tissue during wound healing. The appropriate development of new blood vessels, along with their subsequent maturation and differentiation, establishes the foundation for functional wound neovasculature. We have previously shown that topical application of insulin to mouse wounds accelerates wound closure in vivo and keratinocyte migration and differentiation in vitro. Here we present studies in vivo and used a variety of cellular and molecular approaches in vitro, to show that insulin stimulates angiogenesis and to elucidate the signaling mechanisms by which this protein stimulates microvessel development. Mouse skin injected with insulin shows longer vessels with more branches, along with increased numbers of associated α-smooth-muscle actin-expressing cells, suggesting the appropriate differentiation and maturation of the new vessels. We also found that insulin stimulates human microvascular endothelial cell migration and tube formation, and that these effects occur independently of VEGF/VEGFR signaling but are dependent upon the insulin receptor itself. The signaling pathway activated downstream of the insulin receptor involves the activation of Akt in a PI3K-dependent manner, followed by activation of SREBP and ultimately resulting in activation of Rac1, a GTPase known to be important for cell motility. Inhibition of the various components of this pathway abolishes endothelial cell migration and tube formation in vitro. This signaling pathway appears to operate in insulin-induced angiogenesis in vivo as well; SREBP1 inhibitor 25-hydroxycholesterol inhibited insulin-induced angiogenesis in vivo. These results show the pro-angiogenic effects of insulin and elucidate the signaling pathways stimulated by this protein that result in angiogenesis. Our findings strongly suggest that insulin is a good candidate for the treatment of ischemic wounds and other conditions in which blood vessel development is impaired.

478/B425

Confirmation of MicroRNAs in Zebrafish Hematopoeisis.

J. Maynard[1], S. Lacadie[2], L. Zon[2]; [1]Biology, Howard University, Washington, DC, [2]Children's Hospital, Harvard Medical School, Boston, MA

MicroRNAs are suggested to be regulators of tissue development. The study of hematopoeisis in the zebrafish, Danio rerio serves as a good model since the molecular pathways of hematopoeisis are conserved between mammals and fish. The high fecundity and the external development of the zebrafish embryo makes the model powerful. To study the role of miRNAs in hematopoeisis, LNA-based miRNA microarray expression profiling was done on the zebrafish blood mutant cloche. The Cloche mutant has no blood or vascular tissue, therefore miRNAs downregulated in the cloche mutant are likely to be expressed in these tissues. In Situ Hybridization (ISH) was done to confirm the results of the microarray using special chemically synthesized LNA probes. MiR-144 and miR-451 ISH gave results consistent with the microarray data, showing downregulation in the cloche mutant. Suprisingly, downregulation of the kidney expressed MiR-34 labels it as a possible miRNA involved in blood development

479/B426

Characterization of the EGFR Pathway Using Small Molecule Inhibitors and In-Cell ELISA Technology.

B. L. Webb, D. E. Hughes, J. Narahari; Thermo Fisher Scientific, Rockford, IL

In-Cell ELISA technology represents an emerging new technique for studying intracellular signaling proteins in context of the whole cell using post-translational modification (PTM)-specific antibodies. It offers a convenient and fast cell-based assay methodology for analyzing signal transduction pathways. Typically, Western blots are used to monitor post-translational protein modifications (PTMs), using PTM-specific antibodies. However, Western blotting involves multiple, time-consuming steps, is only semi-quantitative and is very low throughput. PTMs associated with protein activation can be easily monitored by the In-Cell ELISA method in a 96- or 384-well microplate format, allowing for assay scalability and automation while conserving valuable reagents such as cell culture reagents and

stimulators/inhibitors. We used EGFR signaling as a model system to develop and validate reagents for In-Cell ELISA assays. A431 cells were pre-treated with inhibitors targeting various signaling proteins in the EGFR pathway and then stimulated with EGF. Activation of the EGFR pathway was monitored using an In-Cell ELISA assay with phospho-Erk as the endpoint readout. We used both colorimetric and near-IR fluorescent modes of detection. The In-Cell ELISA format allowed analysis of multiple parameters, such as dose and treatment times, in a single 96-well microplate. We demonstrated compound specificity and selectivity in our In-Cell ELISA comparable to traditional Western blotting. Our results suggest that In-Cell ELISA is a convenient and quantitative method for cell signaling studies and drug screening applications.

480/B427

Releasing the Brake on Muscle Growth: Refolding, Purification, and Crystallisation of Mature Human Myostatin.

C. Starck, A. J. Sutherland-Smith; Institute of Molecular BioSciences, Massey University, Palmerston North, New Zealand

The controlled development and maintenance of mammalian skeletal muscle mass is crucial to ensure the correct amount of muscle for a healthy existence. A key player in muscle homeostasis is myostatin, a member of the Transforming Growth Factor Beta (TGFβ) superfamily, whose signalling negatively regulates muscle growth. Over-expression of myostatin leads to muscle atrophy, and absence to an increase in muscle mass, observations that suggest that myostatin inhibition is a promising method to enhance muscle growth in diseases of muscle wastage. Although myostatin inhibitors have been described, the absence of myostatin structural data limits further investigation. This research is focused around the structural analysis of human myostatin by X-ray crystallography. As a TGFβ family member, myostatin is expressed as a precursor protein in vivo and is enzymatically processed to yield the mature, active growth factor dimmer. In contrast to its TGFβ relatives however, mature myostatin has proven extremely difficult to refold with high efficiency when produced as inclusion bodies in E. coli. We describe here the refolding and purification of the myostatin precursor protein and furin cleavage in vitro to yield pure, mature myostatin dimer. Initial crystallisation screens have produced crystals from conditions similar to those used for other TGFβ family members. Although these crystals are not diffraction quality, optimisation is likely to lead to a myostatin structure. This research will not only provide an enhanced understanding of myostatin and thus TGFβ family function and signalling, but will also enable structure-based drug design.

New and Emerging Technologies for Cell Biology I (481-499)

481/B430

Intracellular Manipulation of Single Cells Using Ultrashort Laser Pulses: Mitochondria and Cytoskeleton.

A. Ngezahayo[1], J. Baumgart[2], S. Przemeck,[3], K. Kütemeyer[2], L. Kruppe[1], W. Bintig[1], A. Heisterkamp[2], H. Lubatschowski[2,3]; [1]Institute of Biophysics, Leibniz University Hannover, Hannover, Germany, [2]Laser Zentrum Hannover e.V, Hannover, Germany, [3]Rowiak GmbH, Hannover, Germany

Objective: We investigated the manipulation of mitochondria and other cell organelles in fixed as well as living single cells by optical means and its effect on mitochondrial apoptosis. **Method:** Ultra-short laser pulses were focused by a high numerical aperture objective. The high intensities created within the focal volume enabled visualization and accurate manipulation of cells at a subcellular level. The visualization of the microfilaments as well as the microtubules was achieved by either staining the microfilament with Alexa conjugated phalloidin or labelling actin and tubulin with GFP. Mitochondria were identified with MitoTracker ® Orange and induction of apoptosis was traced by immunofluorescent staining with cytochrome c and Annexin V. **Results:** Disruption of single cytoskeletal filaments as well as mitochondria demonstrated the high precision necessary in order to manipulate cell organelles. Ablation of a single mitochondrion with repetition rates of 4.5 MHz or 45 KHz did not induce cell death. Manipulation of around 20 mitochondria per cell at repetition rates of 4.5 MHz caused swelling of the whole mitochondria population as well as cell membrane blebbing. Release of cytochrome c and positive staining for Annexin V indicated apoptotic changes. **Conclusion:** Intracellular manipulation by optical means offers the possibility to elucidate cellular processes e.g. apoptotic pathways, particular with regard to the mitochondrial pathway, as mitochondria are targets for a variety of apoptosis-inducing drugs in cancer therapy.

482/B431

Remeasuring the Double Helix.

R. S. Fenn[1,2], R. Das[1,3], P. B. Harbury[1,2]; [1]Biophysics, Stanford University, Stanford, CA, [2]Biochemistry, Stanford University, Stanford, CA, [3]Physics, Stanford University, Stanford, CA

DNA is thought to behave as a stiff elastic rod with respect to the ubiquitous mechanical deformations inherent to its biology. We have measured the mean and variance of end-to-end length for a series of DNA double helices in solution, using small angle X-ray scattering interference between gold nanocrystal labels. The data rule out the conventional elastic rod model. Specifically, the variance in end-to-end length follows a quadratic dependence on the number of base pairs rather than the expected linear dependence. Absent applied tension, DNA is at least one order of magnitude softer than measured by single molecule stretching experiments. Our observations indicate that DNA stretching is cooperative over more than two turns of the DNA double helix, and support the idea of long range allosteric communication through DNA structure.

483/B432

A TCSPC Fluorescence Lifetime Microscope System in the Deep UV-Visible and NIR (240nm - 1700nm).

l. Chandler; Molecular & Microanalysis, Horiba Jobin Yvon, Inc., Edison, NJ

Time-resolved fluorescence microscopy is the ultimate tool for investigating dynamic events in cellular/sub-cellular structures and nanomaterials. Applications previously have been limited to wavelengths in the visible region (370-850nm) because microscope optics does not transmit in the deep UV. This paper describes a filter-based confocal system (the DynaMyc™) to measure fluorescence lifetimes and intensity directly under the microscope in the wavelength range of 240-1700 nm for the first time. The system features time-correlated single

photon counting (TCSPC) for sensitive and rapid acquisition of luminescence lifetimes from 100ps to100us. An Olympus BX51 microscope was modified to transmit light for both excitation and emission down to 240nm in the deep UV. To maintain maximum light throughput, both the excitation light source and the detector are directly coupled to the microscope. Excitations are fast pulsed solid state LEDs or laser diodes. The detectors are the fast response TBX detector for UV-VIS (240-850nm) and the Hamamatsu H10330 PMT for NIR (950-1700nm). This system can be used for mapping of fluorescence lifetimes and intensity with variable spatial resolution (>1um) with an automated stage. This paper will present fluorescence lifetime measurement from a single protein crystal with a diameter of 70 um, and lifetime mapping obtained from a stained mouse kidney section. The preliminary data for NIR lifetimes measured from NIR quantum dots will also be presented. The potential application for detection of intrinsic fluorescence from single proteins and deep tissue imaging in the NIR will be discussed.

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Real-Time Imaging of Endocytosis in Vascular Endothelium in Mice Using Fluorescent Receptor-Targeted Nanoparticles and Fluid-Phase Markers.

C. Garnacho[1,2], M. Meng[1], S. Muro[1,2]; [1]Pharmacology, University of Pennsylvania Medical School, Philadelphia, PA, [2]Center for Biosystems Research and Fischell Dept. of Bioengineering, University of Maryland Biotechnology Institute and University of Maryland College Park, College Park, MD

Endocytosis in endothelial cells contributes to cell signaling, communication and migration, blood/tissue homeostasis and host defense. Tracing endocytosis in animal models in real time would help elucidate its regulation and role in physiology and disease. However, binding of labeled-ligands to their receptors at the endothelial surface, followed by their concentration at particular domains, is not optically distinguishable from their uptake and concentration into endocytic vesicular compartments. A method to overcome this obstacle may consist of: 1-Coupling a ligand (or "mimicry ligand" such as an affinity peptide or antibody) to reporter fluorescent polymer nanoparticles, 2-co-injecting the ligand-particles intravenously along with an inert fluid-phase marker that is labeled by another fluorophore, and 3-tracing the passive uptake of the fluid-phase marker into the lumen of endocytic vesicles formed in response to the ligand-particle, revealed by co-localization of both fluorophores. To illustrate this strategy, we absorbed the surface of 100nm- or 1µm-diameter FITC-polystyrene particles with antibodies to intercellular adhesion molecule-1, a surface glycoprotein involved in inflammation, which can mediate endocytosis in endothelial cells. Ligand-particles were injected IV in mice with the fluid-phase marker Texas-red dextran and were traced by fluorescence microscopy in the mesentery vasculature. Ligand-particles flowed in the bloodstream and firmly adhered to the vascular surface 5 min post-injection. At this time, dextran fluoresced diffusely while flowing in the blood (a negative control for dextran interaction with ligand-particles). Shortly thereafter, dextran appeared as a punctate matter co-localizing with ligand-particles, indicating its incorporation into vesicles where the ligand-particles were endocytosed. The vesicles then trafficked forming ring-like structures similar to those observed in cell culture upon transport to perinuclear lysosomes. Endocytosis and lysosomal trafficking were confirmed postmortem by electron microscopy. These preliminary results set the basis to explore this strategy facilitating real-time imaging of endocytic events occurring in vascular endothelium in animal models.

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Nuclear Magnetic Resonance (NMR) Spectroscopy - A Tool for Diagnosis of Malignant and Benign Breast Disorders.

M. Srivastava[1,2], S. Kumar[2], S. Kumar[2], D. K. Gupta[1]; [1]Department of Biochemistry, University of Allahabad, Allahabad, India, [2]Department of Surgery, King George's Medical University, Lucknow, India

Reliable diagnostic tools are being continuously developed to differentiate between benign and malignant breast lesions. We have employed one dimension (1D) and two dimensional (2D) in-vitro Nuclear Magnetic Resonance (NMR) Spectroscopic methods to evaluate metabolic profile of axillary lymph nodes in patients with various breast diseases. Based on number of the metabolites quantitatively estimated from perchloric acid (PCA) extract of tissues, breast tissue samples were classified as malignant, benign and normal by NMR spectroscopic method and markers for predicting pathogenicity were assessed. The sensitivity and specificity of NMR spectroscopy for diagnosis of various breast diseases were obtained by comparing these results to histopathological data. The study was conducted on fifty patients (n=50) with breast disease as evaluated by mammography and biopsy investigations. All samples provided for NMR spectroscopic study were double blinded to detect sensitivity and specificity of NMR spectroscopy. PCA extract of snap frozen breast tissues were subjected to 400 MHz NMR spectrometer. All major metabolites were unambiguously assigned in PCA extract and their absolute concentrations were determined in all cases. Coupled resonances within the aliphatic region of spectra were identified by Double Quantum Filtered - Correlation Spectroscopy (DQF-COSY) and TOtal Correlated SpectroscopY (TOCSY). Significantly high levels of BCA viz. Valine, Leucine & Isoleucine ($p=0.0379$), lactate ($p<0.0001$), Alanine ($p<0.0001$), Glutamate ($p=0.04$), Glutamine ($p=0.0281$), Creatine ($p=0.02$), Choline ($p=0.029$) and Tyrosine ($p=0.0001$) were identified in malignant as compared to benign tissue. Based on study NMR offers 89.47% sensitivity and 100% specificity with 11.76% chance of false positive result for diagnosis of breast diseases. There is clear-cut distinction between extracts of malignant, benign and non-involved breast tissue. Thus, the result provides the potential application of NMR spectroscopy as a diagnostic tool for breast disorders which could be incorporated for future in vivo studies. ACKNOWLEDGEMENTS: The financial support from the ICMR, New Delhi and technical support from CBMR, SGPGI, Lucknow are gratefully acknowledged.

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Measurement of Changes in Phosphorylation of Signaling Proteins in Primary Hematopoietic Malignancies Pre- and Post Therapy Using a Nano-Immunoassay System.

D. Deb-Basu[1], A. Fan[2], D. Voehringer[1], D. Felsher[2]; [1]Cell Biosciences, Palo Alto, CA, [2]Stanford University, Stanford, CA

Protein quantification in clinical specimens is important the development and monitoring of effective therapies that target oncoproteins. Current protein detection methods require large samples, precluding routine serial tumor sampling to assess changes in oncoprotein levels. Changes in the expression and activation of a variety of signaling proteins in malignant hematopoietic (CML) cells and patient samples before and after treatment with therapeutic agents that impact oncogenic signaling pathways are shown by a nano-immunoassay system (NIA-FireflyTM system). Time course studies demonstrated the decrease in phosphorylation of signaling proteins in CML cells after imatinib

treatment. NIA also detected a novel change in the phosphorylation of an ERK2 isomer in patients with CML who responded to therapy. Decrease in STAT3/5 phosphorylation in patients with lymphoma treated with a biological modifier was detected by NIA in a clinical study. A key benefit of this technology is that it separates protein isoforms based on its isoelectric point. Using this assay, we were able to distinguish and quantify the phosphorylated and non-phosphorylated forms of a protein with a single antibody. Thus, we have developed a novel technique which can precisely evaluate the activity levels of signaling proteins in oncogenic pathways from very small samples.

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Neural Cell Adhesion and Neurite Outgrowth on Substrates with Nanostructurally Defined Peptide Density and Tunable Elasticity.

T. Wolfram[1,2], I. Louban[1,2], H. Kessler[3], J. P. Spatz[1,2]; [1]Dept. New Materials and Biosystems, Max-Planck-Institute for Metals Research, Stuttgart, Germany, [2]Dept. of Biophysical Chemistry, University of Heidelberg, Heidelberg, Germany, [3]Institute for Organic Chemistry und Biochemistry, Technical University Munich, Munich, Germany

In the present study, we used a nanoengineered particle array and biofunctionalized the particles with different peptides (RGD from fibronectin, IKVAV from Laminin) to investigate neural cell adhesion and neurite outgrowth. In addition to glass substrates, patterned gold nanoparticle arrays were transferred to elastic polyethyleneglycol hydrogel surfaces, resulting in substrates with different elasticities. Particle density on the nanometer lengthscale, substrate stiffness, and bioactive components could be varied independently from each other. Substrates were characterized with fluorescence-, (cryo)-scanning electron- (SEM), and atomic force microscopy (AFM). Neural cell adhesion of N2a, B35, and SHSY-5Y neuroblastoma cells were investigated on those particle arrays on glass as a function of nanometer lengthscale. All cell types showed similar results with higher cell adhesion on greater nanoparticle densities and lower adhesion on smaller densities, regardless of the biofunctionalization with different peptides. On soft substrates with variations in elasticity from approximately 7 to 15 kPa, cell adhesion for neuronal cells peaked around 10 kPa with significant lower adhesion to substrates with 7 and 15 kPa. In contrast, primary mouse embryonic fibroblasts cells showed on all substrates no significant variations in cell adhesion. Neural cells adhere to both peptides in similar quantities meanwhile fibroblasts adhere preferentially to RGD functionalized substrates. In co-culture assays, more neural cell adhesion was found on 60 nm spaced IKVAV substrates with stiffness values around 10 to 12 kPa. Neurite outgrowth of N2a cells was investigated depending on substrate stiffness and peptide variations. The highest rate for neurite initiation was found on substrates with 15 kPa. The longest neurites were observed at a substrate stiffness of about 10 kPa or lower. In contrast to IKVAV, neurites on RGD substrates showed highest elongation at 15 kPa. In conclusion, neural cell adhesion and neurite formation depends on substrate stiffness as well as biofunctionalization and particle density.

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Well Plate Microfluidic Devices for Image-based Cell Adhesion, Transmigration, and Cell-Cell Communication Assays Under Shear Flow.

C. G. Conant, C. Ionescu-Zanetti, M. Schwartz; Fluxion Biosciences, S. San Francisco, CA

Microfluidic devices have become invaluable tools in recent years to study biological phenomena. Here, we present a well plate microfluidic (WPM) device developed for conducting cell biology assays under shear flow. Physiological shear flow conditions of cell-cell and cell-ligand adhesion within this device produce results with higher biological significance than conventional well plates. The WPM format also produced significant work flow advantages such as faster compound additions. We used the VLA-4- VCAM-1 cell adhesion model as the basis for a rapid, higher-throughput adhesion inhibition screen of monoclonal antibodies against VLA-4. Using the WPM device, we generated IC50 dose response curves 20 times faster than conventional flow cells. The WPM device was also used to study transmigration of mononuclear cells through endothelial cell monolayers. Twenty four channels of transmigration data were generated in a single experiment. A second configuration of the WPM device was used to study epithelial cell wound response. Monolayers of cells were wounded using trypsin and cell migration was tracked following initial wounding. Wound size was found to be uniform using this technique. Wound healing was enhanced with epidermal growth factor and abrogated with cytochalasin D.

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Selective Adhesion and Growth of Astrocytes and Gliomas on Micro Scale Patterns Using Novel Molecular Printing Techniques.

M. A. DeCoster[1,3], D. Green[2]; [1]Biomedical Engineering, Louisiana Tech University, Ruston, LA, [2]Biology, Louisiana Tech University, Ruston, LA, [3]Institute for Micromanufacturing, Louisiana Tech University, Ruston, LA

Interaction with extracellular matrix and/or other cells can induce cell shape changes associated with growth and differentiation. Here, three different cell types were utilized to assess the efficacy of digitally pre-defined fluorescein isothiocyanate labeled poly-l-lysine (FITC-PLL) micro-patterns as templates for promoting spatially defined cell attachment and growth. Patterns composed of FITC-PLL droplets ranging in diameter from 10 to 20 µm with center to center spacing of 30 to 50 µm apart were deposited onto glass substrates using a Nano eNabler molecular printer system and 30 µm wide cantilevers. Substrates were baked to set polymer droplets, sterilized, and seeded with respective cell types at identical starting density. For all cell types, specific association of cells with FITC-PLL patterns was confirmed by fluorescence microscopy combined with nuclear staining of cells. Rat brain astrocytes bound and grew preferentially on printed sub 20 µm diameter patterns, with cells covering greater than 65% of the patterns. Rat brain derived gliomas in control studies were confluent within 24 hours forming a complete monolayer, but on micro-patterns we found that up to 48 hours post plating, glioma cells were preferentially binding and proliferating only along sub 20 µm deposited patterns. In contrast to the above cell types, blood-derived Jurkat T cells bound to patterns, but remained separated from other cells and retained a spherical morphology, due to the printed patterns. Furthermore, T cells became trapped on each FITC-PLL spot, preventing further proliferation of these cancer cells. In separate glioma and astrocyte cultures, single cells initially restricted to patterns continued to proliferate, forming intercellular connections, a marked difference compared to T cell patterned growth. Of the three cell types used, glioma cells demonstrated the fastest growth and proliferation. This work presents a new method for the rapid production of micro-scale printed polymer patterned substrates using femtoliters of material per spot for the study of cellular-level connections, including basic cell-cell communications, cell signaling, and mechanisms of apoptosis, applicable to multiple cell types.

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Growth of Caenorhabditis elegans (C. elegans) on Alternate Food Sources.

K. Szeniawski, L. Brier, F. Norflus; Clayton State Universtiy, Morrow, GA

Lab strains of the bacteria eating nematode C. elegans have been harvested from their natural environments of mushroom beds and compost piles. Labs worldwide maintain stocks of C. elegans on Nematode Growth Medium (NGM) seeded with an OP-50 E. coli bacterial lawn. However, several research papers state that E. coli bacteria are not their natural food source. It is unclear what the natural food source is for C. elegans but scientists have experimented with different strains of bacteria. The objective of this study was to introduce food products (tofu, spinach and mushroom) into the NGM agar medium that may be found in compost piles and mushroom beds. A single C. elegans egg was placed on each plate. Plates were observed for one hatchling. If the egg did not hatch, or if more than one hatchling was observed, the plate was discarded. The growth of the worms from 10 plates was examined for 21 consecutive days. A bacterial lawn was not grown on alternate food source plates. The plates were observed and each was given a number between 0 (no worms observed) and 5 (population was so dense that the surface of the agar could not be seen). The plate population thrived on the alternative food sources supporting the hypothesis that the C. elegans diet may not be restricted to just consuming bacteria. The number of large and small worms on each of the plates was also documented. There was fluctuation during the time course of the study due to the rate of growth of the worms and the fact that the worms were laying eggs during the experiment. However, in general, the alternate food source plates contained more large sized worms while the plates with bacteria as the food source contained more small sized worms.

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Adhesamine, a Newly Synthesized Chemical Compound, Is a Very Useful Substrate for Culturing Mouse Primary-cultured Hippocampal Neurons.

M. Hoshino[1], S. Yamazoe[2], M. Uesugi[2], S. Terada[1]; [1]Neuroanatomy and Cellular Neurobiology, Tokyo Medical and Dental University Graduate School, Tokyo, Japan, [2]Chemical Biology Laboratory, Institute for Chemical Research, Kyoto University, Kyoto, Japan

OBJECTIVE: It is well known that many kinds of cells adhere to plastic or glass surfaces precoated with poly-L-lysine (PLL). PLL is widely used as a substrate for many types of cell culture, including primary-cultured hippocampal neurons. Recently we found a novel chemical compound, named adhesamine, which stimulates attachment of human cells, such as HepG2 and Jurkat cells. Whether adhesamine also exhibits enhanced attachment of mouse primary-cultured hippocampal neurons should be an interesting issue to be examined. METHODS: We examined morphology, differentiation and survival properties of mouse hippocampal neurons grown on either PLL or adhesamine by means of phase contrast microscopy, immunofluorescence, transmission electron microscopy and electrophysiological analysis. RESULTS: Neurons grown on adhesamine-coated coverslips survived for up to 1 month without feeder layer of glial cells, and their viability is higher than that of cells grown on PLL-coated coverslips. Morphological analysis revealed that adhesamine explicitly promotes more prompt differentiation, exemplified by axonal outgrowth, neurite spreading and neurite branching, even at DIV 1. Ultrastructural analysis revealed that cells on adhesamine have characteristic neuronal properties, such as axon differentiation and synapse formation. CONCLUSION: These observations suggest that adhesamine has excellent properties as a substrate suitable for long-term culture of hippocampal neurons. Adhesamine could be a promising substrate for other neuronal cell culture, promoting further cellular neuroscience research.

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A Novel Protein Transduction Method Using the HVJ Envelope Vector System.

Y. Kondo[1], T. Fujieda[1], F. Kato[1], K. Miyata[2], M. Kato[1], Y. Kaneda[3]; [1]Cent. Res. Inst., Ishihara Sangyo Kaisha, Ltd., Kusatsu, Japan, [2]Life Sci Tech. Dev. Div, Ishihara Sangyo Kaisha, Ltd., Osaka, Japan, [3]Div. of Gene Therapy Sci., Osaka Univ. Med. Sch., Osaka, Japan

[Objective] HVJ Envelope (HVJ-E) vector is a non-viral vector prepared making use of the cell-fusing activity of Sendai virus (Hemagglutinating Virus of Japan). We previously reported during a meeting of this society that this vector allows introduction of not only nucleic acids (plasmids, siRNA, etc.) but also proteins into cells while preserving their functions intact. However, the efficiency of incorporating protein into HVJ-E tends to be affected by the type of protein. If this shortcoming can be resolved or alleviated, the scope of application of this vector will be expanded. With this borne in mind, we recently explored methods of delivering protein into cells efficiently, i.e., methods less likely to be affected by the type of protein, by using affinity tags and anti-affinity tag antibody. [Methods] This attempt was based on the technique for efficient incorporation of antibody into HVJ-E we reported during last year's meeting of this society, that is, the method using GenomONE(TM)-CAb kit. We encased anti-affinity tag antibody into HVJ-E and then examined whether or not this HVJ-E could carry fused proteins (conjugated to an affinity tag such as Flag, HA, Myc, etc.) into cells. [Results] This analysis revealed that the use of anti-affinity tag antibody increased the amounts of protein carried with HVJ-E into the cells, as compared to the HVJ-E not encasing the antibody. Furthermore, it was shown that adequate amounts of cationic protein (seemingly difficult to carry with ordinary cationic lipid transfection reagents in view of the principle) could be carried with the HVJ-E vector encasing the antibody. [Conclusions] These results suggest that this system for intracellular transfer of proteins is applicable to a wide range of proteins, while being influenced little by the type of protein. This technique is also promising as a means of screening of inhibitory peptides against intracellular functional molecules, etc., and we are identifying optimum conditions for use of this technique to this purpose.

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Screening Method for Functional Antibodies in Cells Using the HVJ Envelope Antibody Delivery System.

K. Sakai[1], Y. Kondo[1], F. Kato[1], K. Miyata[2], M. Kato[1], Y. Kaneda[3]; [1]Central Research Institute, Ishihara Sangyo Kaisha Ltd., Kusatsu, Japan, [2]Life Sci. Tech. Dev. Div., Ishihara Sangyo Kaish, Ltd., Osaka, Japan, [3]Div. of Gene Therapy Sci., Osaka Univ. Med. Sch., Osaka, Japan

[Objective] HVJ Envelope (HVJ-E) vector is a non-viral vector prepared making use of the membrane-fusing activity of Sendai virus (Hemagglutinating Virus of Japan). During the meeting of this society last year, we reported a method of antibody introduction into cells using HVJ-E. This technique allows efficient delivery of IgG antibody into living cells and is applicable to quantitative evaluation of the function expression level (inhibitory activity) of the antibody bound to the target molecule in the cells. We recently studied a methodology for screening of functional antibodies inhibiting the intracellular signal transduction pathways. [Methods] NFκB, a transcription factor

involved in intracellular signal transduction, served as a model for this study. Using this model, several commercially available antibodies were evaluated as to their activity to inhibit the transcription by NFκB in the following steps: (1) checking antibody reactivity by immunostaining of cells, (2) checking transfer of antibody into cells using HVJ-E (GenomONE(TM)-CAb kit), (3) checking inhibition of NKκB molecular transfer into the nucleus due to the introduced antibody, and (4) checking inhibition of a phenomenon (NO formation) induced by activation of NKκB transcription. The experiment used a mouse macrophage-like cell line Raw264.7. Various anti-NKκB antibodies were encased into HVJ-E and introduced into cells. After LPS stimulation, the inhibitory effect on nuclear translocation of NKκB and on NO formation was evaluated. [Results] When commercially available monoclonal antibodies were evaluated in steps (1) through (4), the antibodies which cleared the steps (1) and (2) but showed suppressed nuclear translocation in step (3) were found to be capable of inhibiting NO formation (step 4) which is the output from signal transduction in this evaluation system. [Conclusions] Evaluation using the basic steps we established was shown to allow efficient selection of antibodies which can exert their functions within cells. The functional antibodies thus selected are applicable to functional analysis and dynamic study of their target molecules.

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Specific Activation of Protein Fluorescence for the Evaluation of Protein Transduction in Live Cells.

Y. Lee, J. Pellois; Biochemistry & Biophysics, Texas A&M University, College Station, TX

Delivery vectors such as Protein Transduction Domains (PTDs) can mediate the translocation of large and hydrophilic macromolecules across biological barriers. Although these tools have proven useful in improving the pharmacodynamics of protein drugs, their mechanism and delivery efficiency have remained a matter of debate. Here, we report a novel method to characterize and quantitatively measure the translocation of cell-permeable proteins across the plasma membrane of live cells. This method combines the use of a protein probe that can respond to its environment by changing its fluorescence output and live cell imaging. Using our approach, we demonstrate that protein translocation can be unambiguously validated, monitored with high spatial and temporal resolution, and precisely quantified.

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Novel Nucleic Acid Stabilization and Enhancement Technologies for PCR Analysis.

P. H. Faix, S. de Rozieres, S. Ohgi, J. Muller-Cohn, R. Muller; Biomatrica, San Diego, CA

We have developed several novel technologies designed to improve upon life science research. One technology stabilizes and protects nucleic acid samples from degradation during storage at room temperature (RT). We have also developed a highly effective PCR enhancer reagent that improves amplification during PCR-based analysis. The objective of this study is to evaluate these technological developments and assess their usefulness in cell and molecular biology research applications. The development of ambient temperature stabilization technologies would provide a useful alternative to current cold storage methods, and has particular application for sample shipments to core facilities. We will also present data on enhancing PCR-based analysis for compromised or limited sample types. The stabilization technology is based on extremophile biology that allows some organisms to survive while in a dry state for >100 years. The synthetic medium forms a thermo-stable barrier during the drying process to protect samples from degradation during storage at RT. Results demonstrate stabilization of DNA for >2 years RT, an equivalent of >14 years under accelerated aging conditions. Bacterial cultures or whole blood samples were also stored in the medium and results indicate stabilization of DNA, even when contained in the unpurified samples. Purified total RNA was successfully stabilized for >8 months at RT and even at 50°C for long time periods. DNA and RNA samples were recovered using a one-step rehydration protocol and used directly without further purification in downstream applications. Data will also be presented demonstrating improved PCR amplification with inclusion of a polymerase enhancer, particularly for compromised and difficult to amplify templates. Studies to evaluate the stabilization of other protein-based samples dry at RT are on-going. Advances in sample preservation that prevent degradation at ambient temperatures and enhanced PCR-based detection will have a significant impact for improving genomic technologies used in biological research.

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New Positive Selection Systems for lacZ beta-Galactosidase and amp beta-Lactamase Transgenic Cells.

A. A. Naleway, T. L. Neff, J. J. Naleway; Research and Development, Marker Gene Technologies, Inc., Eugene, OR

Expression of foreign genes in mammalian cells has become a standard biotechnology protocol, and co-expressed reporter genes are routinely used in these systems to track the expression levels of the simultaneously cloned genes. The complete lack of intrinsic enzyme activity in the resulting recombinant cells and the wide substrate specificities of the enzymes allow use of the marker gene for release of targeted conjugates at very low levels of expression. In an effort to impose a non-destructive selection mechanism on transgenic cells, we have prepared new β-galactoside and Cephalosporin conjugates of common growth regulators, drugs and enzyme activators, for administration to animal cells or bacteria that contain either the beta-galactosidase (*lacZ*) or ampicillin resistance (beta-lactamase, *amp*) genes as gene fusion markers. These new conjugates were used to provide new methods of utilizing these fusion systems in transformed cells to select for transgenic cells in cell culture based upon expression levels of the marker genes. New conjugates of guanosine, L-arginine, L-citrulline, L-phenylalanine, L-leucine, L-isoleucine, L-valine, D-glucose and L-glutamine were prepared and assayed in both *E. coli* and in mammalian skin fibroblast (NIH3T3 and CREBAG2) cell lines grown in cell culture for their ability to cause specific and localized improvement of cell growth and to deliver the active agents in a cell- or co-culture specific manner. By combining the effect of certain metabolic inhibitors including mycophenolic acid (MPA) a GMP (guanosine monophosphate) synthetase inhibitor, or methotrexate, a dihydrofolate reductase inhibitor, with selection media lacking certain amino acids along with administration of a panel of conjugates, an 80-100% selection rate could be effected for lacZ-positive CREBAG2 cells over NIH3T3 (lacZ-negative) after incubation for 3 days in selection media followed by a 7 day recovery period. The use of these methods for selection of transgenic cells lines from progenitor and stem cell lines is currently underway. This work was supported by grant 1R43GM074315-01 from the NIGMS-NIH.

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Detection and Application of Molecular Roadblocks Interfering with Kinesin-1 Based Transport.

T. Korten, S. Diez; Max Planck Institute of Molecular Cell Biology and Genetics Dresden, Dresden, Germany

Biomolecular motors are promising components for highly efficient molecular sorting and nano-assembly devices. In particular, in vitro transport assays usually utilize surface-attached motor proteins to propel cytoskeletal filaments. While motile filaments provide a versatile platform for attachment of transport material, heavy cargo-loading was reported to significantly decelerate gliding motion. However, the details of this effect have remained unclear. To investigate this phenomenon, we performed systematic gliding motility experiments using kinesin-1 motors and biotinylated microtubules loaded with varying amounts of streptavidin. By monitoring microtubule speed in gliding assays, we characterized slow-down as a function of streptavidin concentration on the microtubule lattice. In complementary single molecule stepping experiments we investigated the mechanism behind this cargo-induced deceleration. We found that single kinesin-1 molecules frequently stop when they encounter streptavidin obstacles on the microtubule lattice. However, about half of the stopped kinesin-1 molecules were able to overcome these obstacles eventually. We explain the stopping by kinesin-1's unique properties: Moving processively along individual microtubule protofilaments, kinesin is unable to walk around an obstacle or to detach immediately. However, if the obstacle and kinesin-1 do not occupy the same binding site on the microtubule lattice, molecular flexibility will allow the molecules to pass each other. To demonstrate a novel sensing application, we mixed microtubules labeled either with biotin or rhodamine. Adding streptavidin did then selectively slow down the biotinylated microtubules, while anti-rhodamine antibodies slowed the rhodamine-labeled filaments. We were also able to show that versatile detection via an antigen binding fragment of antibodies is possible. Interestingly, microtubules can then be used for simultaneous detection and transport of arbitrary target proteins. We propose to combine our results with other recent advancements in the field, creating highly integrated lab-on-a-chip devices that use microtubule based transport to detect, sort and concentrate analytes.

498/B447

Isolation and Purification of Drosophila Plasma Membranes.

M. R. Khanna[1,2], G. H. Thomas[1,2]; [1]Biology, The Pennsylvania State University, State College, PA, [2]Biochemistry and Molecular Biology, The Pennsylvania State University, State College, PA

The plasma membrane and its associated proteins play an important role in determining how a cell interacts with those around it as well as how it reacts to components and conditions of its extracellular environment. As a reflection of this, more than 50% of the current drug targets lie on the cell surface. Thus, a characterization of the plasma membrane proteome of a cell would offer insight into not only the functioning of a cell but also in identifying potential drug targets and their response to genetic mutations in pathways that populate the cell surface. However, identification of plasma membrane proteins continues to be a challenge due to their low abundance and poor resolution in 2D gel electrophoresis. We have used a combination of density gradient centrifugation and affinity partitioning in aqueous two-phase systems to isolate plasma membrane from *Drosophila* tissue. Though these two techniques have been used separately for plasma membrane extraction before, we show that with *Drosophila* tissue, a combination of these techniques results in a robust preparation with low-level contamination from other compartments. We have also successfully resolved this plasma membrane fraction by two dimensional electrophoresis and identified membrane proteins by mass spectrometry. In conclusion, we have used a unique combination of established methods to successfully isolate, purify and identify plasma membrane proteins from *Drosophila* . This work is supported by funding from The American Heart Association to Graham H Thomas.

499/B448

New Tools for the Modification and Characterization of Highly Hydrated Pericellular Coats.

H. Boehm[1], T. Mundinger[1], C. Schmitz[1], V. Hagel[1], E. Bock[1], J. Curtis[1,2], J. P. Spatz[1]; [1]New Materials and Biosystems, Max-Planck Institut for Metals Research and University of Heidelberg, Stuttgart, Germany, [2]School of Physics, Georgia Institute of Technology, Atlanta, GA

Many mammalian cells are enveloped by a highly hydrated film of hyaluronan and hyaladherins. The intricate relationship of this so-called pericellular coat (PCC) with the extracellular matrix (ECM) and the cell itself is governed by polymer physics and polyvalent interactions. We are particularly interested in how the polymer mechanics and the structure of the PCC can be influenced. In one approach, nano-patterned substrates are applied to control ECM-cell interaction to study the effect on the intermediate PCC. The resulting mechanical properties of the coat are investigated with microrheology. An important connection between the different ECM components and the cell is established between their RGD motiv. Our nano-patterned substrates offer the possibility to precisely control density and spacing of RGD ligands on an otherwise inert background [1]. As expected chondrocytes (RCJ-P) adhere less to surfaces with larger interparticle distances, evident by smaller adhesion areas and increased motility. Initial results show that the thickness of the cell coat scales inversely with ligand spacing on the nanopattern. In addition to systematically studying the correlation between PCC thickness and ECM-cell interactions, we aim to gather complementary information about the mechanics of the pericellular coat. This requires a measurement technique operating in the micrometer regime. The Brownian motion of sub-micrometer tracer particles enables us to determine the mechanical properties of the embedding solution. We have extended the technique, known as microrheology, for the first time to measure the viscoelasticity of the pericelluar coat on living cells. The micromechanical map shows that HA molecules are arranged around the cell membrane to generate a gradient of increasing stiffness towards the cell membrane, i.e. enabling a non-linear increase of the repulsiveness of the PCC towards objects approaching the cell membrane. [1] M. Arnold, A. Cavalcanti-Adam, R. Glass, J. Blümmel, W. Eck, H. Kessler, J.P. Spatz “Activation of Integrin Function by Nanopatterned Adhesive Interfaces” ChemPhysChem 2004, 5, 383-388.

Imaging Technology I (500-513)

500/B449

Visualization of Individual mRNA Molecule at Single Cell Level in Differentiating Cells.

C. Lai, S. Bui, Q. Nguyen; Panomics, Inc., Fremont, CA

In situ hybridization technique is commonly used to visualize DNA or localized RNA within cells. However the in situ analysis of RNA has always been limited by low sensitivity, complicated workflow, challenging probe synthesis and the inability to perform multiplex analysis. We developed a novel mRNA in situ hybridization technique that offers single-copy mRNA sensitivity in single cells, and is able to detect two target mRNAs simultaneously. With this assay, we profiled the expression pattern of a panel of marker genes in differentiating cells including C2C12 myoblastic cells and 3T3L1 adipocytes. The results demonstrated the profound changes in gene expression profiles in the course of myotube formation and adipogenesis. It also highlighted the differences between undifferentiated and committed cells in the mixed population. This technique has broad applicability in research areas including biomarker validation, as well as verification of in vitro and in vivo RNAi delivery and knockdown. Moreover, the automation-friendly, simple assay workflow is suitable for high-throughput applications such as phenotypic or reporter gene screening.

501/B450

Real Time Monitoring and Quantification of Endogenous mRNA in Single Living Cells.

K. Okabe[1,2], Y. Harada[2,3], T. Funatsu[1,4]; [1]Graduate School of Pharmaceutical Sciences, University of Tokyo, Tokyo, Japan, [2]Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, [3]The Institute for Integrated Cell-Material Sciences, Kyoto University, Kyoto, Japan, [4]Center for NanoBio Integration, University of Tokyo, Tokyo, Japan

In eukaryotic cells mRNA plays a key role in gene regulations. However, the function of mRNA is not fully understood because direct detection of endogenous mRNAs in living cells has been difficult. Here, we developed a novel method for the observation of specific endogenous mRNA in living cells. Endogenous c-fos mRNA was visualized with fluorescently labeled antisense 2'O-methyl RNA oligonucleotides. To detect the target mRNA selectively, we utilized two antisense probes having different fluorophores. These probes are complementary to an adjacent sequence of c-fos mRNA and therefore fluorescence resonance energy transfer (FRET) occurs upon hybridization. Two probes were microinjected into living COS-7 cells and the fluorescence was imaged under an epi-fluorescence microscope. We observed FRET fluorescence and found that endogenous c-fos mRNA was diffusely distributed throughout the cytoplasm of cells. Furthermore, the determination of c-fos mRNA concentration in living cells was performed. Fluorescence correlation spectroscopy (FCS) was employed to quantitatively analyze the kinetics of hybridization of antisense probe and the target mRNA. In living COS-7 cells, two fractions having different diffusion times were observed, suggesting that probes hybridized with mRNA showed slower diffusion times than those of unbound probes. Using a model for hybridization reaction and kinetic parameters such as the Kd value and the concentration ratio of bound/free probe, the concentration of endogenous c-fos mRNA in each COS-7 cell was determined to range from 99.2 nM to 752 nM. This novel method to monitor and quantify endogenous mRNAs in single living cells will help us to elucidate the characteristics of mRNA such as distributions and functions.

503/B452

Localisation of LC3-II, by Confocal and 3D Electron Microscopy, in the Same Cell.

D. Dinsdale[1], M. Rossi[1], S. Bartesaghi[1], J. McWilliam[1], C. Genoud[2]; [1]MRC Toxicology Unit, Leicester, United Kingdom, [2]Gatan Inc, Pleasanton, CA

Autophagy, a term coined in 1963, was largely an ultrastructural concept until a number of autophagy-related genes and their corresponding proteins were identified in yeast. A mammalian homolog of one of these proteins, microtubule-associated protein light chain 3 (LC3-II) localizes to the limiting membrane of autophagosomes and, in conjunction with green-fluorescent protein (GFP), has become the most widespread tool for imaging these organelles. Recent concerns regarding the specificity of this label led us to compare confocal images of LC3/GFP during starvation-induced autophagy, in HeLa cells stably expressing LC3-GFP, with immunogold labelling of both LC3 and GFP on acrylic resin sections of similar cells. The restriction of immunogold to small vesicles and cisternae, rather than the large foci suggested by confocal microscopy led us to process some of the same cells, after confocal imaging, for conventional electron microscopy. Serial sections were prepared by ultramicrotomy and also with the '3-View' sectioning system (Gatan, Inc.) in a 'Quanta 200' environmental scanning electron microscope (FEI, Inc.) and imaged accordingly. Equivalent sections from the respective confocal and ultrastructural 'Z-stacks' were superimposed, enabling the correlation of the fluorescence generated by GFP with the fine structure only visible by electron microscopy. This approach does not avoid problems associated with the permeabilisation of cells for immunolabelling but it does circumvent most of the compromises needed to localise endogenous fluorescent tags at the EM level, enabling the adoption of glutaraldehyde/osmium tetroxide fixation, 'en-bloc' staining and even epoxy resins. The resulting images show that, in this model, LC3 was not incorporated into intracellular protein aggregates, presumably because the LC3 was not overexpressed, e.g. by transient transfection. The large fluorescent foci, evident by confocal microscopy did not, however, prove to be the expected large fused vacuoles but clusters of small vesicles consistent with those observed by immunogold labelling.

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Ht Screening of Biosensor Libraries to Produce Live Cell Fluorescent Biosensors of Endogenous Src Family Proteins.

A. Gulyani[1], E. Vitriol[1], D. Gremyachinskiy[1], B. J. Dewar[1], A. Nguyen[1], L. M. Graves[1], B. Kay[2], K. Hahn[1]; [1]Pharmacology, University of North Carolina, Chapel Hill, NC, [2]Biological Sciences, University of Illinois, Chicago, IL

We present here a new approach to develop biosensors for studying signaling proteins in living cells - including previously inaccessible targets. Biosensors for endogenous cellular proteins often require a binding element that can specifically recognize the active form of the target protein. However, such natural binders are often not available. Here we show that it is possible to use high throughput screening of optimized scaffold libraries to generate binders that can then be used to construct sensitive biosensors using rational design. Phage displayed screening of fibronectin domain III (FN3) monobodies was used to produce binders for Src SH3 domains. We have binders for c-Src, Src family kinases (SFKs) and Lyn. These binders have a strong affinity for their targets, and are highly specific. Our initial focus has been on the SFK binder, named 1F11. Pull-down assays showed that the selected binder (1F11) bound preferentially to the active form of Src, and that biosensor-bound Src kinase retained its activity. Using merocyanine dyes developed for live cell imaging, we generated ratiometric sensors that show a large fluorescence response on binding to the Src SH3 domains, and are bright and sensitive. Our latest sensor versions

are several times brighter than fluorescent proteins commonly used in FRET sensors. Further, the dye based sensors reported here can be used simultaneously with FP FRET sensors for multiplexing. In live cell experiments with mouse embryo fibroblasts (MEFs), we are studying Src activation dynamics in macropinocytic vesicles and membrane ruffles.

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rsCherryRev and NISO Red-shifted Optical Switch Probes for Optical Lock-in Detection (OLID) Imaging and 2-Colour OLID-FRET.

S. Mao, C. Petchprayoon, R. Perrins, G. Marriott; Physiology, University of Wisconsin Madison, Madison, WI

Optical lock-in detection (OLID) microscopy using synthetic or genetically encoded optical switches is was developed by our group to generate high-contrast images of the distributions and interactions of proteins in the presence of high and time-varying background signals such as those found in living cells in culture and in live tissue. OLID requires an optical switch probe whose fluorescence intensity can be modulated through deterministic optical control of its fluorescent and non-fluorescent states. Our initial studies focused on Dronpa, while in this study we show how the genetically-encoded optical switch rsCherryRev can be used to extend the wavelength region of OLID to the red (>550 nm) and for 2-colour OLID imaging in combination with Dronpa. Optical lock in detection (OLID) of Foerster resonance energy transfer, OLID-(FRET) using optical switches as acceptor probes can overcome several limitations of FRET imaging of protein interactions in living cells, including detecting low levels of protein complexes that result from endogenous unlabeled proteins, and non-stoichiometric formation of protein complexes between donor and acceptor probes. We have previously used NitroBIPS as an optically switchable acceptor probe for GFP, and in this study we show how the synthetic, red-shifted optical switch spironaphthoxazine (NISO) serves as a switchable acceptor for both GFP and mCherry in OLID-FRET. These new probes are used for 2-colour OLID-FRET of protein interactions in living cells.

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Spironaphthoxazine (NISO)-derived Optical Switch Probes for Optical Lock-in Detection (OLID) Imaging Microscopy and OLID-FRET.

C. Petchprayoon, S. Mao, R. Perrins, g. marriott; Physiology, University of Wisconsin-Madison, Madison, WI

We present the design, synthesis, and characterization of a new family of optical switch probes for optical lock-in detection (OLID) imaging microscopy and OLID-FRET. These new probes combine the high quantum yield property of tetramethyrhodamine (TMR) probe with the highly efficient optical switching characteristics of the spironaphthoxazine (NISO) probe within a single molecule. NISO undergoes efficient, rapid, and reversible optically-driven transitions between a colorless spiro (SP) state and colored merocyanine (MC) state. The MC state of NISO probe has absorption maximum at 612 nm and serves as an acceptor probe for the excited state TMR in Förster resonance energy transfer (FRET). The fluorescence emission of TMR within TMR-NISO is controlled by optically switching the SP and MC states in NISO resulting in deterministic modulation the TMR quantum yield from 0.8 to zero. The unique and highly optimized features of the TMR-NISO optical switch probe together with the OLID imaging technique is used for imaging low levels of TMR-NISO labeled proteins and cell structures even in the presence of high background signals.

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Automatic Quantitative Characterization of Rapid Protein Dynamics in Live Cell Microscopy Assays.

S. J. Lee[1], S. V. Alworth[1], C. Huang[1], S. Oh[1], H. Watanabe[2], K. Horikawa[3], T. Nagai[3]; [1]DRVision Technologies LLC, Bellevue, WA, [2]Nikon Instruments Company, Yokohama-city, Japan, [3]Research Institute for Electronic Science, Hokkaido University, Sapporo, Japan

Biological reactions depend largely on the diffusion and localization of biomolecules and intracellular organelles. A new generation of microscope and fluorescent probe technologies has enabled the visualization of rapid protein dynamics and molecular and subcellular events. However, quantitative characterization of biomolecule and intracellular organelle mobility is challenging since the dynamics of objects are complex and may be obscured over time. Therefore, much of the kinetic characterization is based on manual tracking and interpretation, which is tedious, subjective and irreproducible. We have developed automatic subcellular object tracking and characterization technologies including 1) a highly robust and flexible tracking method called "soft tracking"; 2) track refinement and state detection; and 3) kinetic characterization. We include new kinetic measurements such as track zones of influence, counts of fast/slow objects, counts of "Association" and "Disassociation" states. The objective of this study is to validate the performance of the technologies using live cell images of photo-convertible, fluorescent protein Phamret (PHotoactivation-Mediated Resonance Energy Transfer) fused to SKL tripeptide for peroxisome localization. Our hypothesis is that our methods achieve similar performance to the best manual method. The manual tracks are created independently by two analysts and discrepancies are resolved through review with the group. We test the hypothesis using detection, tracking, and state detection accuracy metrics. We conclude that our tracking technologies support the hypothesis with statistical significance. We believe the technologies have broad applications, and are working to validate them on a number of live cell assays. This research was funded in part by NIH grant 1R43GM077774-01 awarded to JSJ Lee.

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High Sensitive DIC Microscopy with Contrast Independent of Specimen Orientation.

M. Shribak; Marine Biological Laboratory, Woods Hole, MA

The image in a regular DIC (differential interference contrast) microscope reflects the orientation of the prism shear direction and the optical path gradients in a specimen. If the shear direction lies parallel to the specimen boundary no contrast is generated. Also a bias retardance is generally introduced, which creates a gray background and reduces image contrast. Here we describe DIC technique, which records phase gradients independently of their orientation and with the digitally generated gradient magnitude image free from the gray background. Separate images can show the magnitude distribution of the optical path gradients and of the azimuths, or the two images can be combined into one picture e.g., with the brightness representing magnitudes and color showing azimuths respectively. The orientation-independent DIC data obtained can also be used to compute the quantitative distribution of specimen phase or to generate enhanced, regular DIC images with

any desired shear direction. We investigated various specimens such as Bovine pulmonary artery endothelial cell, human cheek squamous epithelial cell, Sciara coprophila salivary gland polytene chromosomes, Vorticella convallaria, crane-fly (Nephrotoma suturalis) spermatocytes, etc. The OI-DIC system can also be combined with an orientation-independent polarization system. The combined system will yield two complementary phase images of thin optical sections of the specimen: distribution of refractive index gradient and distribution of birefringence due to structural or internal anisotropy of the cell structure. For instance, in a live dividing cell, the OI-DIC image will clearly show chromosomes while the polarization image will quantitatively depict the distribution of birefringent microtubules in the spindle, both without any need for staining or other modifications of the cell. We are currently developing a new device using special DIC prisms, which allows the bias and shear directions to be switched rapidly without the need to mechanically rotate the specimen or the prism. With the new system an orientation independent DIC image should be obtained in a fraction of a second.

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Chemical Imaging of Lipid Distribution within Phase-separated Supported Lipid Membranes.

M. L. Kraft[1], P. K. Weber[2], P. Juristyarini[1], C. R. Anderton[1], K. Lou[1], I. D. Hutcheon[2]; [1]Chem. & Biomol. Engr., University of Illinois, Urbana, IL, [2]Glenn T. Seaborg Institute, Lawrence Livermore National Laboratory, Livermore, CA

Elucidating the lateral distribution of components within the cell membrane and the effect of this organization on biological processes is an area of much interest. Phase-separated supported lipid bilayers are frequently used to investigate the molecular interactions that underlie cell membrane organization. Though these model systems enable precise control of the lipid species within the bilayer, imaging variations in the lateral composition of a model membrane is still a major challenge. We previously reported that the distributions of two isotopically labeled lipids in a phase-separated supported lipid bilayer can be imaged and quantified with a lateral resolution of 100 nm using multi-isotope imaging mass spectrometry (MIMS). MIMS performed with the NanoSIMS 50 (Cameca Instruments) reveals the elemental and isotopic composition in a sample with a lateral resolution as high as 50 nm. By incorporating a distinct stable isotope into each membrane component of interest, the isotopically enriched secondary ions produced by the labeled molecules reveal their identities. Here we use this method to visualize the distribution of cholesterol within a phase-separated supported lipid membrane. The lipid bilayer was composed of a 13C-labeled lipid, a 15N-labeled lipid, and 18O-labeled cholesterol (CHO-18O). At room temperature, phase separation occurs between the 13C-labeled lipid and the 15N-labeled lipid, but the partitioning of CHO-18O between these two lipids cannot be directly visualized. MIMS analysis revealed that the CHO-18O was nearly equally distributed between the domains that were enriched by the 13C-labeled lipid or the 15N-labeled lipid. By further expanding on this method, direct visualization of the component distributions within actual cell membranes might be realized.

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Automated Quantitative Analysis of Montaged Confocal Datasets Using FARSIGHT.

C. Bjornsson[1], C. Tsai[2], Y. Al-Kofahi[2], A. Narayanaswamy[2], K. Smith[3], D. LeBlanc[3], W. Shain[3], B. Roysam[2]; [1]Center for Biotechnology & Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, [2]Department of Electrical, Computer, and Systems Engineering, Rensselaer Polytechnic Institute, Troy, NY, [3]Wadsworth Center, New York State Department of Health, Albany, NY

The brain's reactive responses to implantation of neural prosthetic devices involve numerous cell types across a large volume of tissue. Automated image analysis methods are crucial to developing quantitative measures of brain responses to neural prosthetic devices. Montaged confocal image stacks generate massive data sets, especially as researchers begin to investigate responses to larger devices (e.g. DBS electrodes). We present recent advances in FARSIGHT (Fluorescence Association Rules for image inSIGHT) that enable us to derive quantitative information from montages that span large territories across multiple tissue slices. Using this approach we can chart the interrelationships among cells that are several hundred microns or even millimeters apart in order to generate a comprehensive map of tissue architecture. As an example, 80µm-thick rat coronal sections were immunohistochemically labeled to highlight cell nuclei, neurons (Nissl), astrocytes (GFAP), microglia (Iba1), and blood vessels (EBA). A montage of 60 images beginning at the cortex and extending into hippocampus was used to demonstrate FARSIGHT's ability to montage across serial tissue sections. A set of 23 intrinsic and 8 associative image-based measurements were computed for each cell. Objects were grouped using a fuzzy c-means clustering algorithm, from which a training set was derived. This set was edited manually, and used to compute a support vector classifier that enabled us to classify cells. A joint generalized dual-bootstrap registration algorithm was used to transform each of the 60 datasets into a common frame of coordinates. Roughly 30,000 cells were visualized using 3-D color-coded graphics rendering. The cytovascular map was analyzed statistically to identify outliers that were inspected manually and edited when appropriate. Additional validation was performed on a stereological basis. Cell classification accuracy ranged from 81 - 92%. Accompanying the map is a detailed XML file of measurement data that was queried to identify cell layers, generate quantitative summaries describing the spatial associations among neurons and microglia by cell layer, and compute distributions of cellular spatial relationships to the vasculature.

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Biarsenical Labeling of Tetracysteine-tagged Proteins after Chemical Fixation.

G. Gaietta[1], K. Henry[1], T. J. Deerinck[1], S. R. Adams[3], R. Tsien[3,4], M. H. Ellisman[1,2]; [1]National Center for Microscopy and Imaging Research, Center for Research on Biological Structure, UC San Diego, La Jolla, CA, [2]Department of Neurosciences, UC San Diego, La Jolla, CA, [3]Department of Pharmacology, UC San Diego, La Jolla, CA, [4]Howard Hughes Medical Institute, UC San Diego, La Jolla, CA

The biarsenical/tetracysteine tagging system (Griffin, B.A. et al., Science, 1998; Adams, S.R. et al., J. Am. Chem. Soc., 2002) presents several advantages for live cell imaging. The small tetracysteine motif minimizes the possibility of disruptive incorporation into the host protein. The biarsenical compounds are small, membrane permeable and become brightly fluorescent upon binding to the target tetracysteine. The ability to label with biarsenicals in chemically fixed samples would add to the versatility of this system, for example by eventually providing a means to stain deep tissues from virally transfected or transgenic animals. We employed two separate cell lines, one stably expressing a Golgi-targeted GFP fusion protein and another stably expressing a gap junction protein GFP fusion, both tagged with the tetracysteine peptide FLNCCPGCCMEP (Martin, B.R. et al., Nat. Biotechnol., 2005). Biarsenicals effectively labeled methanol fixed cells,

but aldehyde fixatives almost completely abolished biarsenical binding, presumably because aldehydes react with thiols. Exposure to 2,2'-pyridyldisulfide to protect thiols as disulfides prior to fixation, followed after fixation by NaBH4 to destroy residual aldehydes and triethylphosphine to regenerate thiols, restored labeling with biarsenicals. We found the procedure to be effective both at room temperature and at 4°C - maintaining the sample at the lower temperature results in improved ultrastructure for exploration at the electron microscopic level. Samples thereby prepared can be further labeled with specific antibodies and/or subjected to fluorescent photoconversion of diaminobenzidine (DAB) for correlated light and electron microscopy studies (Gaietta, G. et al., Science, 2002; Gaietta, G. et al., PNAS, 2006).

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Three-Dimensional Structure of the Lantern Organ of the Firefly Photinus Studied by Array Tomography and Scanning Block Face Electron Microscopy.

R. Portugues[2], J. Buchanan[1], K. Micheva[1], S. J. Smith[1]; [1]Molecular & Cellular Physiology, Stanford University, Stanford, CA, [2]Molecular & Cellular Biology, Harvard University, Cambridge, MA

We used two recently developed techniques, array tomography (Micheva and Smith, 2007) and scanning block face electron microscopy (SBFSEM, Denk et Horstmann, 2004) to study the molecular architecture of the light producing lantern organ of adult Photinus fireflies captured in Woods Hole, Massachusetts. Array tomography uses multiple antibodies to simultaneously label thin (70- 250 nm) serial sections of LR White embedded tissues, which are bonded onto glass microscope slides. The study of immunolabeled thin sections allows imaging with unprecedented resolution (< 300nm in the x-y plane and <100nm in the z-direction) using fluorescence microscopy and leads to informative 3D volume reconstructions of serial section data. Antibodies and fluorescent probes we used include markers for luciferase, nitric oxide synthase, chitin, mitochondria, nerve endings, octopamine, glutamate and tyrosine hydroxylase. We were able to provide detailed localization of these molecules in the different cell types within the lantern organ: photocytes, tracheolar cells, tracheolar end cells and tracheolar epithelial cells. In addition, we performed electron microscopy, TEM and scanning block face electron microscopy (SBFSEM)(Gatan, USA), to obtain ultrastructural details of the anatomy of the lantern organ. We focused in particular on the regions where the tracheolar end cells terminate in mitochondrial rich regions and searched for nerve endings. Both array tomography and electron microscopy suggest that nerve endings are not on the photocytes but instead on the tracheolar cells. In conclusion, by using these two new imaging techniques, we provide detailed anatomical structure and molecular architecture of the lantern organ to improve understanding of the mechanisms of flash control in the adult firefly, a subject of much debate (see Trimmer et al. 2001, Timmins et al. 2001 or Ghiradella and Schmidt 2004 for three different proposed mechanisms).

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Nanoscale Imaging of Cochlear Hair Cells Using Hopping Probe Ion Conductance Microscopy.

P. Novak[2], C. Li[3], A. Shevchuk[2], R. Stepanyan[1], D. Klenerman[3], Y. Korchev[2], G. Frolenkov[1]; [1]Department of Physiology, University of Kentucky, Lexington, KY, [2]Division of Medicine, Imperial College of Science, Technology and Medicine, London, United Kingdom, [3]Department of Chemistry, Cambridge University, Cambridge, United Kingdom

Current methods to image the complex three dimensional cell surfaces at high resolution require fixation or freezing, which introduces artifacts and does not allow direct visualization of functionally important processes in live cells. Here we present a major advance in scanning probe microscopy that allows the imaging of live complex cellular samples without ever touching them. We demonstrate the effectiveness and nanoscale resolution of the technique by imaging well-organized mechanosensory stereocilia of the cochlear hair cells. Our technique can be straightforwardly combined with fluorescence detection to identify functionally important structures in these networks. Because the same nanoprobe can be used not only for imaging, but also for local stimulation of surface nanostructures and/or probing their electrical activity, our technique opens up a possibility for structure-function studies at a nanoscale. This study was supported by the Kentucky Science and Engineering Foundation (#KSEF-148-502-07-215) and the National Organization for Hearing Research Foundation (to GF) and by the Biotechnology and Biological Sciences Research Council (to YK).

Gene Structure and Expression I (514-531)

514/B465

Primary microRNA Transcript Retention at Sites of Transcription Leads to Enhanced microRNA Production.

J. Pawlicki[1], J. Steitz[2]; [1]Pharmacology, Yale University, New Haven, CT, [2]Molecular Biophysics and Biochemistry, Yale University, New Haven, CT

MicroRNAs (miRNAs) are noncoding RNAs with important roles in regulating gene expression. miRNAs are initially transcribed by RNA polymerase II as much longer primary miRNA (pri-miRNA) transcripts. Pri-miRNAs are cleaved in the nucleus into stem-loop precursor miRNAs (pre-miRNAs) by the RNase III-like enzyme, Drosha, and its essential cofactor, DGCR8. Pre-miRNAs are then exported and further processed to mature miRNAs in the cytoplasm. Recent studies have revealed that pri-miRNA processing is regulated. During mammalian development and in several diseases including cancer, many pri-miRNA transcripts are expressed but are not processed to pre- or mature miRNAs; the mechanisms underlying this regulation remain unclear. The objective of this study is to better understand miRNA biogenesis and its regulation. We observe that primary miRNA transcripts retained at transcription sites due to the deletion of 3′-end processing signals are converted more efficiently into pre-miRNAs than pri-miRNAs that are cleaved, polyadenylated, and released. Flanking exons, which also increase retention at transcription sites, likewise contribute to increased levels of intronic pri-miRNAs. Consistently, efficiently processed endogenous pri-miRNAs are enriched in chromatin-associated nuclear fractions. In contrast, pri-miRNAs that accumulate to high nuclear levels after cleavage and polyadenylation due to the presence of a viral RNA element (the ENE of the Kaposi ' s sarcoma - associated herpes virus polyadenylated nuclear RNA) are not efficiently processed to precursor or mature miRNAs. These results suggest that pri-miRNAs undergo initial processing at transcription sites, and that pri-miRNA processing may be enhanced by coupling to

transcription. Furthermore, because several pre-messenger RNA processing events, including splicing and 3′-end formation, occur and are regulated cotranscriptionally, regulation of pri-miRNA processing may also be coordinated with transcription.

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A Role for the snRNP Repertoire in Splicing Regulation.

D. Kaida, K. Dittmar, Z. Zhang, I. Younis, L. Wan, M. Kasim, G. Dreyfuss; Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia, PA

The survival of motor neurons protein (SMN) is essential for the biogenesis of small nuclear ribonucleoproteins (snRNPs), the major constituents of the pre-mRNA splicing machinery (the spliceosome). Though it is ubiquitously expressed, SMN deficiency causes the most common motor neuron degenerative disease, spinal muscular atrophy (SMA). As a result of SMN deficiency SMA patient cells have a correspondingly reduced capacity for snRNP assembly. We have recently shown [Zhang et al, Cell 133: 585-600 (2008)] that SMN deficiency alters the stoichiometry of snRNPs and causes widespread pre-mRNA splicing abnormalities. These surprising findings established a key role for the SMN complex in RNA metabolism and in splicing regulation and suggested a role for the snRNP repertoire in determining splicing patterns. To address this we modulated the functional levels of several of the snRNPs of the major (U1, U2, U4/U6, and U5) and minor (U11, U12, U4atac, U6atac, and U5) spliceosomes and determined precise changes in splicing using exon microarrays. The observed splicing changes reveal a major role for the snRNP repertoire in global regulation of splicing and provide important insights into the mechanism of intron recognition.

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Molecular Characterization of SRrp38, A Novel Splicing Regulator Located in the Nucleolus and Nuclear Speckled Domain.

P. Ouyang; [1]Anatomy, Chang Gung University, Guei-San, Taiwan, [2]Molecular Medicine Research Center, Chang Gung University, Guei-San, Taiwan

Objective: We report here the identification and characterization of a novel SR-related protein, referred to as SRrp38, based on its apparent molecular size and subcellular location. Methods: SRrp38 was identified through a yeast-two hybrid screen during the course of searching for proteins interacting with pNO40, a ribosomal 60S core subunit. Subcellular localization of SRrp38 was carried out with monoclonal antibodies directed against endogenous protein. In vivo splicing assay was conducted with reporter minigene in combination with cellular transfection. Results: SRrp38 exhibits two alternative spliced isoforms generated by differential usage of translation start site with the longer one, SRrp38, initiates at first exon while the shorter SRrp38-2 initiates at exon 2. Three distinct motifs can be discerned in the SRrp38 protein: a serine-arginine (SR) dipeptide enriched domain, a poly-serine stretch, and a potential nucleolar localization signal comprising bipartite sequence of basic amino acids. SRrp38 message is expressed in different tissues in distinct ways. In pancreas, prostate, testis and brain, both SRrp38 and SRrp38-2 are highly expressed and at comparable levels. Neither message is produced at significant levels in other tissues examined. Indirect immunofluorescence analysis with an anti-SRrp38 antibody, as well as an experiment using a myc-tagged protein, demonstrated that SRrp38 was localized in the nucleolus and the nuclear speckle. With adenovirus E1A and chimeric calcitonin/dhfr construct as a splicing reporter minigene, SRrp38 was demonstrated to be able to modulate alternative 5' and 3' splicing in vivo. Conclusions: SRrp38 is an uncharacterized novel protein with splicing regulation function that has not been described in recent spliceosome proteome. Studies on this novel splicing regulator may provide new information on the intricate splicing machinery as related to the RNA metabolism involving processing of mRNA or/and rRNA.

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Sequence Similarity to a Donor Splice Site: What Are the Evolutionary Consequences?

K. C. Keller, D. E. Pumo; Biology, Hofstra University, Hempstead, NY

Within a pre-mRNA sequence, the donor splice site, (A,C)AGGU(A,G)AGU, identifies the 5' exon-intron boundary. This consensus sequence does not have to be strictly followed for spliceosome recognition, in fact most naturally occurring donor splice sites deviate from this consensus at two or three positions. Messenger RNA sequences that only slightly deviate from this consensus donor splice site encode short amino acid segments that differ depending on the reading frame and the specific nucleotides used. Acquisition of a single nucleotide mutation can transform one of these amino acid sequences into a donor splice site. Because the activation of cryptic splice sites is documented to cause various pathologies such as Hutchinson-Gilford progeria syndrome, Brugada syndrome, and neurofibromatosis, we asked the question whether the amino acid combinations whose underlying mRNA sequences closely resemble a donor splice site, are evolutionarily unfavorable. Computer programs were designed to analyze a dataset containing approximately 37,000 human proteins. This dataset was derived from the translation of refseq mRNA sequences downloaded from NCBI. The three six-fold degenerate codon classes were separated into two-fold and four-fold classes because of their differential matching to a donor splice site. We found that the mRNA combinations containing a serine, arginine, or leucine, (6-fold degenerate codons) showed a preference for the two-fold or four-fold codon class which conferred a sequence which was least like a donor splice site. The remaining amino acid combinations, which did not contain a serine, arginine or leucine, were present in the dataset at higher than expected levels. This indicates that there may be a selective advantage for maintaining a sequence that closely adheres to a donor splice site despite the increased likelihood of a mutation activating a cryptic splice site. A possible explanation may be that these sequences are serving as a pool of potential locations for the generation of novel alternative splice sites.

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The Histone Acetyl Transferase PCAF Associates with Actin and hnRNP U for RNA Polymerase II Transcription.

A. Obrdlik[1], A. Kukalev[1], E. Louvet[1], A. Östlund Farrants[2], L. Caputo[1], P. Percipalle[1]; [1]Department of Cell and Molecular Biology, Karolinska Institute, Stockholm, Sweden, [2]Institute of Cell Biology, The Wenner-Gren Institute, Stockholm University, Stockholm, Sweden

In the eukaryotic cell nucleus, actin and actin-associated proteins have been implicated in gene transcription bound to the RNA polymerase (pol) machinery. In pol II transcription, actin in complex with a subset of hnRNPs has been suggested to function in the recruitment of

transcriptional co-activators such as histone modifying enzymes during nascent mRNA transcript elongation. Using a combination of affinity chromatography, protein-protein interaction assays and biochemical fractionation of nuclear extracts we discovered that the HAT PCAF associates with actin, hnRNP U and the hyperphosphorylated pol II. Upon disruption of the actin-hnRNP U interaction with a novel peptide-specific antibody (CED17) against the hnRNP U actin binding site, both hnRNP U and PCAF were released from the nuclear actin complex isolated by DNase I affinity chromatography. When performing HAT activity assays on the nuclear actin associated proteins, disruption of the actin-hnRNP U interaction also promoted specific release of the corresponding HAT activity. Consistent with their association with hyperphosphorylated pol II, actin, hnRNP U and PCAF were co-precipitated from nuclear extracts with recombinantly expressed pol II C-terminal domain (CTD) phosphorylated on Ser2/5 and Ser2 within the conserved heptapeptide repeats. Chromatin (ChIP) and chromatin RNA immunoprecipitations (RIP) revealed that actin, hnRNP U and PCAF are present at promoter and coding region of constitutively expressed pol II genes and they are associated with nascent RNP complexes. Finally, in vivo disruption of the actin-hnRNP U interaction by nuclear microinjection of the CED17 antibody repressed BrUTP incorporation in newly synthesized RNA in a HAT dependent manner. Our results are consistent with a cooperative action between the actin-hnRNP U complex and the PCAF complex along active genes during ongoing transcription. We propose a model where the actin-hnRNP U interaction is required to recruit the HAT PCAF to active genes for efficient elongation of nascent RNA transcripts.

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RNA-binding Protein HuR Interacts with Thrombomodulin 5'UTR and Represses IRES-mediated Translation under IL-1β Treatment.

J. T. Tseng; Institute of Bioinformatics, Tainan, Taiwan

Reduction in host activated protein C levels and resultant microvascular thrombosis highlight the important functional role of protein C anticoagulant system in the pathogenesis of sepsis and septic shock. Thrombomodulin (TM) is a critical factor to activate protein C in mediating the anticoagulation and anti-inflammation effects. However, TM protein content is decreased in inflammation and sepsis, and the mechanism is still not well defined. In this report, we identified that the TM 5'UTR bearing the internal ribosome entry site (IRES) element controls TM protein expression. Using RNA probe pull-down assay, HuR was demonstrated to interact with the TM 5'UTR. Overexpression of HuR protein inhibited the activity of TM IRES, while on the other hand, reducing the HuR protein level reversed this effect. When cells were treated with IL-1β, the IRES activity was suppressed and accompanied by an increased interaction between HuR and TM 5'UTR. In the animal model of sepsis, we found the TM protein expression level to be decreased while concurrently observing the increased interaction between HuR and TM mRNA in liver tissue. In summary, HuR plays an important role in suppression of TM protein synthesis in IL-1β treatment and sepsis.

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Translocated Promoter Region Protein Regulates c-jun Transcription through Interaction with a cis-regulatory Element Spanning -538 to -514 Region of c-jun.

S. Agarwal, A. Dixit; School of Biotechnology, Jawaharlal Nehru University, Delhi, India

Jun oncoprotein, a major component of transcription factor AP-1 is responsible for expression of multiple genes which trigger cell proliferation, differentiation and apoptosis. To obtain a conclusive insight into c-jun induced cell transformation, it is important to unveil the molecular mechanisms leading to an enhanced expression of c-jun itself. Transient transfection using series of stepwise deletion of the upstream region linked to basal *c-jun* promoter in a reporter plasmid revealed the existence of a 25 bp region (-538 to -514) responsible for an enhanced c-jun expression. The *trans*-acting factor(s) from rat liver nuclear extract interacting with this region were identified, binds in phosphorylated form and possessed high affinity and specificity. UV-crosslinking, South-Western blotting in conjunction with affinity purification analysis revealed that two protein(s) of 45 kDa and 34 kDa specifically bind this region. Appearance of an 80 kDa adduct in UV-crosslinking suggests that both these proteins interact with recognition sequence. Characterization of 35 kDa protein revealed it to be a component of the nuclear pore complex-attached intranuclear filaments, translocated promoter region protein (Tpr) and 42 kDa protein was identified as β-actin. Co-transfection studies further substantiated direct role of Tpr in augmenting *c-jun* transcription. Spectroscopic tools were employed to establish the DNA-binding potential, stoichiometery of interaction and dissociation constants for Tpr and β-actin. Chromatin immmunoprecipitation provided evidence to the in-vivo DNA-binding potential of both the proteins. Tpr and β-actin also interacted with each other and thereby a model has been proposed for *c-jun* transcriptional activation. Tpr, a natively unfolded protein attained conformational stability upon DNA binding. Identification of Tpr as a protein involved in modulating *c-jun* transcription may facilitate discovery of drugs, as this can be targeted to specifically downregulate c-jun expression during pathophysiological conditions.

521/B472

Post-transcriptional Regulation of cPLA2α mRNA Expression under IL-1β Treatment in A549 Cells.

W. Liao[2], J. T. Tseng[1,2]; [1]Institute of Bioinformatics, National Cheng-Kung Univ., Tainan, Taiwan, [2]Institute of Biosignal Transduction, National Cheng-Kung Univ., Tainan, Taiwan

cPLA2 α is the major intracellular form of PLA2, which selectively hydrolyzes membrane phospholipids at the sn-2 position, and is the rate-limiting enzyme in the eicosanoid production. In our previous study, we found that IL-1β, a pro-inflammatory cytokine, significantly increase the expression of cPLA2α mRNA and protein in A549 cells. However, the promoter activity of cPLA2α gene stimulated by IL-1β only increases around 1.4 fold. Therefore, we were interested in elucidating whether another mechanism involved in co-regulating the expression of cPLA2α. In addition to transcriptional regulation, the post-transcriptional regulation of mRNA turnover has emerged as a key step in the regulation of eukaryotic gene expression. Although the mechanisms determining mRNA turnover are not well established, they are generally believed to be involved in RNA-binding proteins recognizing specific RNA sequences. By cloning the 3'-UTR of cPLA2 α into the reporter vector, we found that the reporter activity was down-regulated. These results indicated that this region contains cis-elements that are known to confer the mRNA instability. From the RNA-EMSA assay, we located the cis-element and this region contains a lot of AU-rich elements (ARE). Furthermore, we identified that HuR can bind to this region as demonstrated by the antibody-shift assay. The same result was also

obtained from in vitro binding assays when we used the GST-HuR fusion protein to perform the experiments. In addition, we observed that IL-1β enhanced the endogenous binding between HuR and cPLA2α mRNA as seen from the RNA-IP assay. Finally, the siRNA technology to knockdown HuR was used, and found that the expression of cPLA2α induced by IL-1β was inhibited. Based on these results, we conclude the notion that HuR plays an important regulatory role in stabilizing cPLA2α mRNA.

522/B473

The Interferon-stimulated Gene Factor 3 Complex Mediates the Inhibitory Effect of Interferon-β on Matrix Metalloproteinase-9 Expression.

X. Zhao, S. Nozell, Z. Ma, E. N. Benveniste; Cell Biology, University of Alabama at Birmingham, Birmingham, AL

Matrix metalloproteinase-9 (MMP-9) displays a preference for a broad range of substrates including extracellular matrix proteins and cytokines. MMP-9 plays an important role in physiological processes, as well as in inflammatory diseases and numerous cancers. Interferon-β is a pleiotropic cytokine with antiviral, antiproliferative and immunomodulatory activities. Interferon-β positively regulates gene expression, predominantly through the Janus kinase-signal transducer and activator of transcription (STAT) pathway. However, little is known about the mechanisms used by interferon-β to negatively regulate gene expression. In the present study, we show that interferon-β inhibits MMP-9 gene expression at the transcriptional level. Using cell lines deficient in three components of the interferon-β-activated interferon-stimulated gene factor 3 (ISGF3) complex (i.e. STAT-1, STAT-2 and interferon regulatory factor 9), the results of our study indicate that all three members are required for interferon-β inhibition. Chromatin immunoprecipitation assays demonstrate that interferon-β reduces recruitment of transcriptional activators and coactivators, such as nuclear factor kappa B p65, Sp1, CREB-binding protein and p300, to the MMP-9 promoter, and decreases the degree of histone acetylation at the MMP-9 promoter. This occurs in the absence of an association of the ISGF3 complex with the MMP-9 promoter. Taken together, these data define the role of interferon-β and the ISGF3 members in suppressing MMP-9 gene expression.

523/B474

Deletion Analysis of the Human Cytochrome Oxidase Subunit IV Isoform 1 Promoter.

A. Sullivan, N. J. Bachman; Biology Department, SUNY College at Oneonta, Oneonta, NY

Cytochrome oxidase is a critical enzyme of the mitochondrial respiratory chain that transfers electrons from cytochrome *c* to molecular oxygen. The enzyme complex consists of numerous subunits; subunit IV is the largest nuclear-encoded subunit which regulates enzyme activity in response to cellular oxygen levels and energy demands. Our objective is to identify regions of the human cytochrome oxidase subunit IV isoform 1 promoter (*COX4I1*) essential for gene expression. Previous studies demonstrated that human *COX4I1* is controlled by a 250 bp bidirectional promoter. In this project, we created three non-overlapping 50 bp deletions in the *COX4I1* promoter by site-directed mutagenesis. We then tested the effects of the deletions on *COX4I1* expression in human cervical cancer cells (HeLa cells). Each of the three deletion mutants reduced *COX4I1* expression to about one fifteenth to one thirtieth that of the intact promoter. However each retained about 3-6 fold more transcriptional activity than that of a minimal promoter construct. The deletion mutations encompass several putative regulatory elements, including two binding sites for NRF-2/GABP, and one for ATF/CREB.

524/B475

HDAC1/HDAC3 Modulates PPARG2 Transcription through the Sumoylated CEBPD in Hepatic Lipogenesis.

J. Wang; Ins. of Biosignal Transduction, NCKU, Tainan, Taiwan

CCAAT/Enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptors gamma (PPARG) play critical roles in the regulation of lipid metabolism genes. Overexpression of CEBPdelta (CEBPD) enhances lipid accumulation and specifically activates PPARG2 transcription in HepG2 cells. By using 5'-serial deletion reporter analysis, we show that the region comprising the -457 to +129 base pairs is required for CEBPD response of the PPARG2 promoter. Two critical CEBPD-binding motifs on the -324/-311 and -158/-145 of human PPARG2 promoter are identified. We previously have shown that the human CEBPD is sumoylated by small ubiquitin-related modifier-1 (SUMO1). We further demonstrated that the sumoylation of CEBPD lysine 120 is also detectable in HepG2 cells, and this modification functions for binding of the recruits, HDAC1 and HDAC3. Meanwhile, an in vivo chromatin IP assay demonstrated that the sumoylation mutant of CEBPD lost a significant portion of HDAC1 and HDAC3 interaction. Combining that the increasing amount of CEBPD and the sumoylated CEBPD (suCEBPD) consistently responded to lipogenic stimulation, these results suggest that the excess non-sumoylated CEBPD could be a critical activator which reverses suCEBPD/HDAC1/HDAC3-mediated PPARG2 gene inactivation and promotes hepatic lipogenesis. Taken together, we suggest that suCEBPD/HDAC1/HDAC3 complex inactivates PPARG2 transcription, and the induction of CEBPD expression transiently activates PPARG2 transcription which is involved in adipocyte-like lipogenesis in HepG2 cells.

525/B476

Redox Regulation of Activator Protein-1-dependent Sulfiredoxin Gene Expression in Macrophages.

Y. Jung[1], B. Shin[1], H. Kim[1], H. Kim[1], H. Song[1], W. Jeong[2,1]; [1]Life and Pharmaceutical Science, Ewha Womans University, Seoul, South Korea, [2]Life Science, Ewha Womans University, Seoul, South Korea

Sulfiredoxin (Srx) is an enzyme being responsible for reduction of cysteine sulfinic acid of peroxiredoxins (Prxs), especially 2-Cys Prxs (Prx I-IV). Here we showed that Srx expression was upregulated transcriptionally by lipopolysaccharide (LPS) in murine bone marrow-derived macrophages (BMM) and RAW264.7 cells. Among putative NF-κB and activator protein-1 (AP-1) sites found in the upstream region of Srx gene, proximal three AP-1 sites were required for LPS-induced Srx expression and were nuclear targets of c-Jun and c-Fos. AP-1 activation is induced by two mitogen-activated protein kinases, c-Jun N-terminal kinase (JNK) and p38, of which JNK played a major role in Srx induction by LPS. In addition, antioxidants such as N-acetylcysteine and butylated hydroxyanisole, and the NADPH oxidase (Nox) inhibitor diphenyleneiodonium inhibited LPS-induced Srx expression. Moreover generation of reactive oxygen species (ROS) and Srx induction by

LPS were suppressed in BMM cells lacking Nox2. These results suggest that in macrophages stimulated with LPS, Srx expression is induced by AP-1 activation through JNK pathway, to which Nox2-derived ROS may be contributed.

526/B477

Epidermal Growth Factor-activated ARNT/HIF-1β Signal Pathway Up-regulates Cyclooxygenase-2 Gene Expression Associated with Squamous Cell Carcinoma.

B. Chen[1], K. Chang[2], M. Shen[1], M. Lee[1], W. Wang[1], W. Su[1], W. Chang[1]; [1]National Cheng Kung University, Tainan, Taiwan, [2]National Health Research Institutes, Tainan, Taiwan

Aryl hydrocarbon receptor nuclear translocator (ARNT), also known as hypoxia-inducible factor (HIF)-1β, acts a pivotal role in tumor growth under hypoxic conditions together with HIF-1α. HIF accumulates when tumors grow under hypoxic conditions. However, the genesis of tumors usually starts from normoxic conditions. In this study, we were interested in examining the potential role of ARNT/HIF-1β in tumor growth under normoxic conditions, specifically when cells were treated with epidermal growth factor (EGF) that is known to affect the gene expression of tumor growth-related protein cyclooxygenase-2 (COX-2). The results showed that EGF receptor inhibitor, AG1478, abolished EGF-induced nuclear accumulation of ARNT as well as the expression of COX-2. ARNT small interfering RNA (siRNA) inhibited the promoter activity, mRNA level, and protein expression of COX-2 in cells treated with EGF. In contrast, CoCl2-induced HIF-1α exhibited no effect on COX-2 expression. EGF also stimulated the formation of the ARNT/c-Jun complex as well as the complex binding to the COX-2 promoter. ARNT siRNAs blocked EGF-activated cell migration. Moreover, COX-2 and ARNT were cohorts present distinctively in clinical specimens of human cervical squamous cell carcinoma and were almost non-detectable in adjacent normal or noncancerous cervical tissues. Our results revealed that ARNT plays an important role in EGF-regulated COX-2 gene expression and may thus be related to either a cause or a consequence of tumorigenesis in cervical cancer.

527/B478

Analysis of Cyclooxygenase-1 and -3 Expression in Prostate Carcinoma.

M. Raya, S. M. Paul, B. A. Jackson; MassBay Community College, Wellesley Hills, MA

The recent identification of Cyclooxygenase-3 (COX3), a splice variant of Cyclooxygenase-1 (COX-1), has originally been considered for pharmacological purposes. Still, there is no clear information on whether this splice variant serves a particular purpose or if it is just an splice error. Through this study we try to analyze the expression of both, COX-1 and COX-1, to compare the ratios of expression under different circumstances; showing that this ratio is not constant would hint us to an specific role for these novel enzyme. To this end we will use two prostate carcinoma cell line (PC3 and DU145) and lipooxygenase A2, a well known unspecific stimulator of COX-1 and COX-2 expression. We will measure the expression using RT-PCR due to is sensitivity compared to Northern blot.

528/B479

Epigenetic Gene Silencing of Werner Syndrome Gene in Hepatocellular Carcinoma Cell Lines.

S. Lee[1], E. Son[1,2], N. Park[1], B. Ahn[1,2]; [1]Ulsan University Hospital Biomedical Research Center, Ulsan, South Korea, [2]Department of Biological Sciences, University of Ulsan, Ulsan, South Korea

Werner syndrome gene (WRN) that was identified as a gene responsible for Werner Syndrome plays essential roles in cellular events including DNA repair, DNA replication, and telomere maintenance. Defects in WRN cause a genetic disorder of premature aging and are closely associated with a high incidence of cancer. Thus, WRN has been considered as a caretaker of the genome and tumor suppressor. However, the precise link between a deficiency of WRN and tumorigenesis is still unclear. We analyzed the hypermethylation of CpG island promoter for WRN from 11 hepatocellular carcinoma (HCC) cell lines using the methylation-specific PCR, and found that six cell lines out of eleven HCC had both methylated DNA and unmethylated alleles. Interestingly, five cell lines expressed a low level of WRN in both RNA and protein level even though they had only unmethylated DNA. Our present study suggests that the methylation status of CpG island of WRN and WRN expression may be associated with hepatocellular carcinogenesis.

529/B480

Expression Analysis of Chondrocytes Seeded on Scaffolds upon Mechanical Stimulation via Continuous Ultrasound.

G. Hasanova, T. Mamedov, A. Subramanian; University of Nebraska-Lincoln, Lincoln, NE

Low-intensity ultrasound (US) has been considered to be an effective method to induce cartilage repair after injury. Previously several reports have addressed the effect of US on the activity of cultured chondrocytes *in vitro* in monolayer or 3D scaffolds; however, the mechanism by which chondrocytes sense and react to US stimuli is not fully understood. Our results showed that chondrocytes when stimulated with continuous US for predetermined time intervals had higher levels of type-II collagen, aggrecan, Sox5 and Sox9 mRNA expression when compared to controls. However, in the same condition the expression of MMP-3, a matrix degradation protein that is important in matrix integrity, was decreased. Interestingly, at the level of expression both Sox5 and Sox9 genes are coordinately responsive to changes of US treatment, and generally mirror the response of Collagen type-II transcript abundance to changes of US treatment. These data suggest that, perhaps upon US stimulation, up-regulation of Collagen type II is Sox9 dependent. We also show that US stimulation impacts the "inside-out" expression of cell-surface integrins and the gene expression of integrins α2, α5, αV and β1 alters with the frequency of US stimulation. While α5 and β1 were up-regulated, α2 was significantly down-regulated at higher application frequency of US stimulation, supporting the idea that many kind of integrins are probably involved in the regulation of chondrocytic function in response to US stimui. In conclusion, taken together our results demonstrate that the exact mechanism of action of US is complex and many genes and pathways, especially signaling pathways are involved in cell response to US effect in chondrocytes.

530/B481

Analysis of CMG2 Expression in Normal and Cancerous Human Prostate.

M. Vargas, G. Chaudry; Biology, South Texas Center for Emerging Infectious Diseases, University of Texas at San Antonio, San Antonio, TX

Capillary morphogenesis gene 2 (CMG2) encodes a single-pass type-1 integral membrane protein. It was first identified by differential gene expression analysis following induction of capillary-like tubulation in collagen matrices by human umbilical vein endothelial cells. CMG2 has therefore been suggested to be important for biological processes requiring vasculature formation. These processes include normal as well as pathological states, such as normal tissue development, wound healing, and tumor development. Moreover, certain mutations in CMG2 have been associated with two early age disorders -infantile systemic hyalinosis and juvenile hyaline fibromatosis. CMG2 protein interacts with collagen (VI) and laminin. However, it is unclear whether these interactions have functional consequences. Aside from these interactions, the only definitively known function of CMG2 is that it functions as an anthrax toxin receptor, a non-native role. The protein has multiple splice variants that show selective tissue-specific and developmental expression in an array of normal human tissues. But their expression patterns in cancerous tumors are not known. We have carried out expression analysis of all CMG2 splice variants in paired normal and cancerous prostate tissues. For this analysis we employed semi-quantitative nested PCR, and analyzed samples from a number of patients. Our analysis has revealed that two integral membrane forms, CMG2-488 and CMG2-489, exhibit near ubiquitous expression in both normal and tumor tissues. In sharp contrast, however, the third membrane-bound form, CMG2-386, showed no expression at all. CMG2-322, a secreted form, showed selective expression; it was present in nearly half the cancer samples and absent from the rest. Next, we plan to analyze the expression of corresponding CMG2 proteins in the same tissues. This would help define more precisely the expression patterns, and would help discern any patterns that correlate with normal or the cancer phenotype.

531/B482

Expression of AP-2delta in the Developing Chick Retina.

X. Li, D. Glubrecht, R. Godbout; University of Alberta, Edmonton, AB, Canada

AP-2 is a family of transcription factors that play important roles during embryonic development. Two AP-2 genes, AP-2alpha and AP-2beta, have previously been characterized in chick retina. Expression of AP-2alpha and AP-2beta is restricted to two neuronal cell lineages; amacrine (AP-2alpha and AP-2beta) and horizontal cells (AP-2beta). We have recently found that a third member of the chicken AP-2 family, AP-2delta, is primarily expressed in the retina and brain. By in situ hybridization and immunohistochemical analysis, we show that AP-2delta RNA and protein are found in a subset of ganglion cells in embryonic chick retina. Co-immunostaining with anti-Brn3a and anti-AP-2delta antibodies indicates that approximately one-third of Brn3a-positive ganglion cells express AP-2delta. AP-2delta RNA but not AP-2delta protein is also found in cells located in the outer half of the inner nuclear layer. The spatial and temporal distribution of AP-2delta protein in the retina suggests a role in a subset of late-born ganglion cells likely involving axonal trafficking or pathfinding. Identifying the target genes of AP-2delta in the developing retina and studying the consequence of AP-2delta mis-expression during development will uncover the functional role(s) of this transcription factor in the developing retina.

Nuclear Architecture (532-546)

532/B483

Farnesyltransferase Inhibitor Treatment Causes Donut-shaped Nuclei.

V. L. Verstraeten[1,2], L. A. Peckham[1], M. Olive[3], C. MacGillivray[1], B. C. Capell[4], F. Collins[4], E. G. Nabel[3], C. L. Stewart[5], L. G. Fong[6], S. G. Young[6], J. Lammerding[1]; [1]Medicine, Brigham & Women's Hospital, Harvard Medical School, Cambridge, MA, [2]Dermatology, University Hospital Maastricht, Maastricht, Netherlands, [3]National Heart, Lung, and Blood Institute, National Institutes of Healths, Bethesda, MD, [4]National Human Genome Research Institute, National Institutes of Healths, Bethesda, MD, [5]Institute of Medical Biology, Proteos, Singapore, [6]Medicine, David Geffen School of Medicine, University of California, Los Angeles, Los Angeles, CA

Farnesyltransferase inhibitors (FTIs) were initially developed as anticancer drugs targeting Ras oncogenes. Recently, FTIs have been proposed as a treatment for Hutchinson-Gilford progeria syndrome (HGPS), a progeroid disorder caused by mutations in the gene for the nuclear envelope protein lamin A. The mutant lamin protein in HGPS causes misshapen nuclei and increased nuclear stiffness, and both of these abnormalities are improved by FTI treatment. However, anecdotal observations suggest that FTIs cause donut-shaped nuclei. **Objective & Methods:** To quantify the frequency of donut-shaped nuclei in FTI-treated cells and to identify the mechanisms underlying "donut formation" and the effects of this morphological abnormality on cell function, we treated human skin fibroblasts from HGPS patients and healthy control subjects with FTI or vehicle alone and then evaluated nuclear morphology and cellular function. FTI-induced donut formation was monitored by time-lapse videomicroscopy of fibroblasts expressing GFP-tagged lamin A. **Results:** FTI treatment caused donut-shaped nuclei in ~5-10% of cells, both in fibroblasts from HGPS patients and normal controls. Donut-shaped nuclei were also observed in lamin A-deficient fibroblasts, suggesting that this morphological abnormality is mediated by some farnesylated protein other than lamin A. The "donut-holes" were free of chromatin, delineated by a nuclear lamina, and contained cytoskeletal filaments and mitochondria. We also identified donut-shaped nuclei in intestinal, skin, and blood vessel tissue sections of FTI-treated mice. Time-lapse videomicroscopy revealed that donut-shaped nuclei are generated during nuclear envelope reassembly following mitosis. Blocking cell division with either serum starvation or nocodazole eliminated donut formation. Cells with donut-shaped nuclei were not susceptible to either apoptosis or cellular senescence, and we found no evidence of DNA damage in donut nuclei. However, even though we observed BrdU incorporation into donut-shaped nuclei, very few of these cells completed mitosis. **Conclusion:** FTI treatment can result in donut-shaped nuclei *in vitro* and *in vivo*. Cells containing donut nuclei are viable but have mitotic defects.

533/B484

Signal-dependent Induction of PML NBs and Regulation of Transcription by Histone Deacetylase 7 (HDAC7).

C. Gao[1], X. Cheng[1], C. Ho[2], E. Reineke[1], M. Lam[3], K. Stanya[1], S. Chakraborty[1], Y. Liu[1], Q. Liu[1], H. Shih[2], K. Chang[4], H. Kao[1]; [1]Department of Biochemistry, Case Western Reserve University, Cleveland, OH, [2]Institute of Biomedical Science, Academia Sinica, Taipei, Taiwan,

[3]Ireland Cancer Center, Case Western Reserve University, Cleveland, OH, [4]Department of Molecular Pathology, University of Texas MD Anderson Cancer Center, Houston, TX

Promyelocytic leukemia protein (PML) nuclear bodies (NBs) are dynamic sub-nuclear compartments that play roles in several cellular processes including apoptosis, transcriptional regulation, and DNA repair. Histone deacetylase 7 (HDAC7) is a potent corepressor that inhibits transcription by MEF2 transcription factors. We show here that endogenous HDAC7 and PML interact and partially co-localize in PML NBs. Tumor necrosis factor alpha (TNFα) treatment recruits HDAC7 to PML NBs and enhances association of HDAC7 with PML in human umbilical vein endothelial cells (HUVECs). Consequently, TNFα promotes dissociation of HDAC7 from MEF2 transcription factors and the promoters of MEF2 target genes such as matrix metalloproteinase-10 (MMP-10), leading to accumulation of MMP-10 mRNA. Conversely, knockdown of PML enhances the association between HDAC7 and MEF2 and decreases MMP-10 mRNA accumulation. Accordingly, ectopic expression of PML recruits HDAC7 to PML NBs and leads to activation of MEF2 reporter activity and siRNA knockdown of PML decreases basal and TNFα-induced MMP-10 mRNA accumulation. PML sumoylation has been proposed to control the formation of PML NBs and is crucial for PML-dependent cellular processes. However, the regulatory mechanisms of PML sumoylation and its specific roles in the formation of PML NBs remain largely unknown. We show that HDAC7 knockdown reduces the size and the number of the PML NBs in HUVECs. HDAC7 coexpression stimulates PML sumoylation independent of its HDAC activity. Furthermore, HDAC7 associates with the E2 SUMO ligase, Ubc9, and stimulates PML sumoylation in vitro, suggesting that it possesses a SUMO E3 ligase-like activity to promote PML sumoylation. In summary, our data support a model in which TNFα controls cell fate, partly by promoting the association between PML and HDAC7 to regulate expression of a subset of HDAC7 target genes and to stimulate PML sumoylation and NB formation.

534/B485

Nuclear Scaling in Xenopus Egg Extracts and Embryos.

D. L. Levy, R. Heald; Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Cell sizes differ depending on developmental stage, cell type, and organism. Little is known about how nuclear size is appropriately regulated relative to cell size. We are utilizing extracts prepared from eggs of two related *Xenopus* species, as well as from developing *X. laevis* embryos, to characterize and elucidate intrinsic mechanisms of nuclear scaling. We find that nuclei assembled *in vitro* using *X. laevis* egg extract are larger than nuclei assembled with egg extract isolated from the smaller related frog *X. tropicalis*. Strikingly, intermediate sized nuclei form when the two extracts are mixed, indicating cytoplasmic factors, rather than genome content, regulate nuclear size. We have also investigated nuclear scaling during early *X. laevis* embryogenesis when rapid cell divisions generate a midblastula transition (MBT) embryo (stage 8) made up of a few thousand cells that have a 25-fold smaller diameter than the initially one mm egg. Despite this dramatic change in cell size, we find that nuclear area decreases only modestly (250 to 125 μm^2) from the 8-cell to the MBT embryo. By stage 10 and later, nuclear size is less than 50 μm^2, and a constant nuclear-to-cell size ratio is achieved. To address whether nuclear transport is involved in nuclear scaling in eggs and embryos, we measured import kinetics of a GFP reporter fused to a nuclear localization signal (NLS) and found a positive correlation between nuclear protein import and nuclear size, with larger nuclei importing more GFP-NLS with faster kinetics. Using embryo extracts prepared at different developmental stages, we show that small nuclei isolated from later stage embryos scale larger when exposed to early stage extract, and that this growth is dependent on nuclear import. Interestingly, nuclear pore complex (NPC) density is greater in embryonic nuclei from earlier stages, but is comparable in *X. laevis* and *X. tropicalis* egg nuclei. Our results suggest that multiple mechanisms impacting nuclear transport contribute to nuclear scaling.

535/B486

The Nucleus Self-Organizes According to Coordinate Gene Regulation.

S. Kosak; [1]Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA, [2]Cell and Molecular Biology, Northwestern University School of Medicine, Chicago, IL

Current evidence indicates that chromosomes are non-randomly localized within the nucleus. Various chromosomal attributes, including gene density, size (Mbp length), and co-regulated gene activity, have been indicated in their organization in mammalian nuclei. Given this growing evidence for deterministic nuclear organization, identifying its origin and structure are important questions. We have previously argued that coordinate gene regulation during cellular differentiation is both responsible for and informed by a cell-specific nuclear topology that facilitates gene co-regulation. We now suggest that a full understanding of nuclear organization necessitates the view that it is a dynamical system, manifested spatially at the level of chromosomal organization such that chromosomes associate according to their overall co-regulation. To test this hypothesis we have used mathematical modeling of datasets that analyze total gene regulation and the spatial arrangement of all chromosomes along a time course of differentiation. Our study focuses on distance matrices that measure the change in entropy of both gene expression and chromosome proximity. We have determined that cellular potential decreases as gene regulatory order increases. Furthermore, we demonstrate that this change in relative gene expression is correlated with the overall proximity of chromosomes that contain these genes. Finally, we show that that the relationship between coordinate gene regulation and chromosome proximity result in nuclear self-organization.

536/B487

Lamin A and Telomerase: A pas de deux of Cell Senescence?

K. Cao, F. Collins; NHGRI/NIH, Bethesda, MD

The classic Hutchinson-Gilford progeria syndrome (HGPS), a devastating premature aging disease, is caused by a point mutation in the lamin A gene (LMNA). This mutation constitutively activates a cryptic splice donor site in exon 11, resulting in a mutant lamin A protein with an internal deletion, termed "progerin". In cell culture, progerin acts as a dominant negative form of lamin A to disrupt nuclear structure and interfere with normal mitosis, limiting cell viability. Recent studies have demonstrated that an identical lamin A splicing event occurs in normal human cells and tissues at low levels, implying that the same mechanisms responsible for HGPS may contribute to normal aging. It remains unknown, however, whether the use of the cryptic splice site in normal cells is constant, or whether this is affected by the aging

process. To answer this question, we directly compared the frequency of the activation of the cryptic splice site in early and late passage fibroblasts from the same individual. Use of the cryptic splice site increased with cell passage, suggesting that senescence induces more progerin expression. Screening various primary and transformed cell lines revealed an interesting inverse correlation between the length of telomeres and the amount of progerin mRNA. Furthermore, down-regulation of telomerase in cancer cell lines by siRNA knockdown resulted in increased progerin mRNA. Our findings suggest a possible synergism between telomere shortening and progerin expression in induction of cell senescence, and increase the likelihood that the study of rare patients with HGPS can teach us something fundamental about the molecular basis of normal aging.

537/B488

Sensing of Curved Membranes by Nup133 Is Critical for Nuclear Pore Complex Assembly.

C. Doucet, M. Hetzer; The Salk Institute for Biological Studies, La Jolla, CA

In eukaryotic cells, the nucleoplasm and cytoplasm are separated by the nuclear envelope (NE), a double lipid bilayer composed of the inner (INM) and outer nuclear membrane (ONM). Local fusions of the INM and ONM generate aqueous channels that are occupied by the nuclear pore complexes (NPCs), which provide the only gateways for nucleo-cytoplasmic transport. The NPCs are disassembled and reformed during each cell division. The conserved multiprotein Nup107/160 subcomplex, a scaffolding component of the NPC, is essential for the NPC assembly process. One of its members, Nup133, has been shown to contain an ALPS domain, which is an amphipathic helix featuring specificity towards highly-curved membranes. In this study we show that the ALPS domain of Nup133 is indeed critical for the proper localization of the Nup107/160 subcomplex to new assembly sites at the NE and for NPC assembly. The disruption of the ALPS domain, by deletion or single mutation, which inhibited membrane-sensing activity of Nup133, resulted in relocalization of Nup107/160 complex proteins from the NE to the cytoplasm in cultured cells. Furthermore, replacement of Nup133 by its ALPS mutants inhibited the recruitment of multiple nucleoporins to the NE and reduced pore density. We propose a model in which the ALPS domain of Nup133 is crucial for the proper targeting of the Nup107/160 subcomplex to the sites of membrane fusion, which is in turn a critical step for NPC assembly.

538/B489

Probing Functions for Son in Nuclear Speckle Structure and Gene Expression.

A. Sharma[2,1], S. Varia[2,1], A. Bubulya[1], P. Bubulya[2]; [1]Biological Sciences, Wright State University, Dayton, OH, [2]Biomedical Sciences Ph.D. Program, Wright State University, Dayton, OH

In mammalian cells, nuclear speckles are storage sites for the assembly of pre-mRNA processing machinery. However, specific mechanisms for nuclear speckle assembly and regulation of pre-mRNA processing machinery within these compartments are not fully understood. Proteomic analysis of nuclear speckles revealed 33 novel speckle proteins including Son1. Son is a large protein (2564 amino acids), and one-third of its sequence is comprised of six types of novel repeat motifs that are not found in any other known proteins. The repeat structure of Son suggests that it may have a molecular scaffolding role in nuclear speckles. Our study aims to determine nuclear functions for Son in structural organization of speckles as well as in pre-mRNA transcription and splicing. We have generated rabbit polyclonal antibodies against both the amino- and the carboxy-termini of Son and demonstrated colocalization of endogenous Son with splicing factor 2/alternative splicing factor (SF2/ASF) in nuclear speckles. Interestingly, depletion of Son in Hela cells that are treated with Son siRNA oligos causes a significant reorganization of nuclear speckles from their typical compact and irregular shape into an open donut-like shape, and this phenotype can be rescued by expression of exogenous siRNA-resistant Son. In addition, cell cycle analysis of Son-depleted cells shows an increase of cells in G2/M phase. The altered organization of nuclear speckles and impeded cell growth pattern observed after Son depletion suggests that Son plays a novel role in the structural maintenance of nuclear speckles and is essential for normal cell cycle progression.

539/B490

FBXO25-associated Nuclear Domains: A Novel Subnuclear Structure.

A. O. Manfiolli[1], A. Maragno[1], M. Baqui[2], S. Yokoo[1], F. R. Teixeira[1], E. Oliveira[1], M. Gomes[1]; [1]Biochemistry and Immunology, Faculty of Medicine of Ribeirão Preto, Ribeirão Preto, Brazil, [2]Cellular and Molecular Biology, Faculty of Medicine of Ribeirão Preto, Ribeirão Preto, Brazil

Skp1, Cul1, Rbx1 and the FBXO25 protein form a functional ubiquitin ligase complex. Here we investigate the cellular distribution of FBXO25 and its colocalization with some nuclear proteins using immunochemical and biochemical approaches. FBXO25 was monitored with affinity-purified antibodies raised against the recombinant fragment spanning residues 2 to 62 of the FBXO25 sequence. FBXO25 protein was expressed in all mouse tissues tested except striated muscle, as indicated by immunoblot analysis. Confocal analysis revealed that the endogenous FBXO25 was partially concentrated in a novel dot-like nuclear domain that is distinct from clastosomes and other well-characterized structures. These nuclear compartments contain a high concentration of ubiquitin conjugates and at least two other components of the ubiquitin-proteasome system: 20S proteasome and Skp1. We propose to name these compartments FANDs for FBXO25-associated nuclear domains. Interestingly, inhibition of transcription by actinomycin D or heat shock treatment drastically affected the nuclear organization of FBXO25-containing structures, indicating that they are dynamic compartments influenced by the transcriptional activity of the cell. Also, we present evidences that an FBXO25-dependent ubiquitin ligase activity prevents aggregation of recombinant polyglutamine-containing huntingtin protein in the nucleus of HEK293 cells, suggesting that this protein can be a target for the nuclear FBXO25 mediated ubiquitination.

540/B491

Ultrastructural Changes of the Neuronal Cell Nuclei in the Rat Dentate Gyrus upon Development of Epilepsy.

A. Szczepankiewicz[1], E. Wilczek[2,1], M. Malinowska[1], G. M. Wilczynski[1,2]; [1]Nencki Institute, Warsaw, Poland, [2]Medical University of Warsaw, Warsaw, Poland

Gene expression is considered to be a fundamental mechanism underlying both normal and pathological neural plasticity, including that involved in epileptogenesis. Although the molecular mechanisms of transcription and mRNA processing in neurons are deeply understood,

little is known about the relationship between these processes and the architecture of the neuronal cell nucleus. Thus, there is a need to characterize in detail nuclear ultrastructural changes associated with various aspects of neuronal plasticity. The aim of the present project was to evaluate the ultrastructure of the rat dentate gyrus (DG) granule cell nuclei in animals suffering from kainate-induced epilepsy. DG granule neurons are known to be resistant to kainate-induced degeneration, instead they undergo profound plastic changes associated with axonal sprouting and synaptogenesis. The study was performed in 6 epileptic male Wistar rats, 6 month after status epilepticus, and in age-matched controls. The ultrastructural analysis demonstrated changes in the appearance of the DG granule neuronal nuclei, associated with epileptogenesis. Most prominently, there was a chromatin clumping accompanied by clearing of the interchromatin space, and an appearance of the large clusters of interchromatin granules (ICG) therein. The relative volume of ICG clusters increased significantly ($p<0.0001$) from 3.5±0.02% in the control group to 6.1±0.02% in the experimental group. Our study indicates that epileptogenesis may be accompanied by a marked modification of higher-order organization of the neuronal nucleus.

541/B492

Roles of the Tudor Domain of Lamin B Receptor for Chromatin Condensation.

Y. Hirano[1], K. Hizume[1], Y. Suzuki[1], H. Takahashi[1], T. Horigome[2], K. Takeyasu[1]; [1]Graduate School of Biostudies, Kyoto University, Kyoto, Japan, [2]Graduate School of Science and Technology, Niigata University, Niigata, Japan

Nuclear envelope (NE) regulates various chromatin dynamics. Recent finding that heterozygous mutation of lamin B receptor (LBR), which is one of the inner nuclear membrane proteins, is caused by nuclear abnormality such as disorganization of heterochromatin structure and nuclear lobulation lead us to investigate the role of LBR in chromatin organization. Although, its regulatory mechanism is still unclear, the amino acids corresponding to 1-89 have been suggested to bind directly to chromatin. Especially, amino acids corresponding to 1-62 constitute a tudor domain that is proposed to recognize a specific histone code. In this study, we investigated a role of the tudor domain of LBR. Fluorescence recovery after photobleaching (FRAP) analysis on deletion mutants of LBR revealed that deletion mutants of chromatin binding domain, $LBR^{\Delta 1\text{-}53}$ and $LBR^{\Delta 1\text{-}89}$, moved 7.7- and 11.2-times faster than the wild type (diffusion coefficiency: 0.030±0.002 $\mu m^2/s$), respectively. The diffusion coefficients of point mutants in the tudor domain, W16A, Y23A, Y41A and D43A, became 4.8-8.9 times faster, indicating that these residues are important for the LBR dynamics in the NE. *In vitro* binding assay using GST-fused W16A showed that this single mutation greatly decreased the histone-binding ability of the tudor domain. Atomic force microscopy on the chromatin-tudor domain interaction revealed that the wild type promoted a chromatin condensation, whereas W16A mutation canceled the chromatin condensation ability. These results suggest that a histone-binding activity of the tudor domain of LBR participated in chromatin organization under the NE, and that this ability is a factor to limit the LBR dynamics in the NE.

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pNO40/pS1D, a Ribosome Associated Protein, Functions as a Nucleolar Recruiter of SR Family Splicing Factors and Impairs poly(A)+ mRNA Nuclear Export.

Y. Zhen[1,2], P. Ouyang[1,2]; [1]Anatomy, Chang Gung University, Kwei-Shan, Taiwan, [2]Molecular Medicine Research Center, Chang Gung University, Kwei-Shan, Taiwan

Objective: Ribosome assembly in nucleolus and nuclear export of poly(A)+mRNA is a crucial aspect on regulation of gene expression. Studies aimed at elucidating the ribosome assembly in nucleoli and poly(A)+ mRNA nuclear export, we characterized that the pNO40 is a novel ribosomal protein being dynamics between different nuclear bodies, and involving in regulation of ribosome biogenesis. Methods: Yeast two hybrid and co-immunoprecipitation is used to confirm the protein-protein interaction. The Sv40-CAT reporter mingene assay was used to analyze RNA transcription. The endogenous GFP fused pNO40 construct was employed for time lapse observation. Results: The pNO40 is originally identified as the pnn binding partner by the yeast two hybrid screening. Further yeast two-hybrid analyses using either pnn or pNO40 as a bait, we found protein SRrp130 could interact with pnn and pNO40, respectively. Co-immunoprecipitation experiment corroborated the yeast two-hybrid data, suggesting the formation of a ternary complex among pnn, pNO40 and SRrp130 .Over-expression of pNO40 induced its self aggregation within the nucleoli and recruitment of certain SR and SR related proteins, including ASF, SC35 and Pnn, into nucleoli from nuclear speckled domains in a time-dependent fashion, a scenario can be demonstrated by the partition of these proteins into triton-insoluble fraction along with a nucleolar marker, fibrillarin. To determine the domain of pNO40 responsible for its oligomerization within nucleoli and recruiting SR proteins into nucleoli, various pNO40 mutants were constructed. Our results revealed that two nuclear localization signals located in the carboxyl basic domain of pNO40 are necessary and sufficient for self-aggregation and SR proteins recruitment within nucleoli. Finally, we demonstrated that pNO40 over-expression impairs pre-mRNA procession and bulk polyA+ mRNA nuclear export through a SV40-CAT reporter minigene assay and fluorescence in situ hybridization. Conclusion: pNO40 is a ribosomal protein interacting with SR and SR-related protein and involve pre-mRNA splicing regulation.

543/B494

Hypertonic Incubation of Living Cells Reversibly Challenges the Integrity of the Nuclear Periphery.

M. Nessling[1], K. Richter[2], H. Herrmann[1], P. Lichter[2]; [1]Functional Architecture of the Cell B065, German Cancer Research Center (DKFZ), Heidelberg, Germany, [2]Molecular Genetics B060, German Cancer Research Center (DKFZ), Heidelberg, Germany

We recently observed, by fluorescence and electron microscopy, that hypertonic treatment of living cells shifts nuclear organization towards compaction of chromatin and segregation of other nuclear compounds into structurally distinct domains (Richter et al. (2007) J Cell Sci 1: 1673-1680). Of particular interest, in-vivo incubation of cells at about 560 mOsm osmotic load provoked a fundamental re-organization of the nuclear architecture: The peripheral chromatin compacted and detached from the nuclear lamina, giving rise to a new chromatin-free compartment, the 'peripheral layer'. At the ultrastructural level, it comprised an outer layer of several hundred nanometers thickness with fine-fibrillar texture, and a heterogeneous array of miscellaneous focal structures at the interphase with the retracted chromatin. Among those focal structures, small clusters of interchromatin granules (i.e. SC35-positive speckles) were identified. Together with similar phase-separation processes throughout the nucleus, these changes could be attributed to effects of macromolecular crowding. When transferred back into isotonic growth medium, all of the observed hypertonic re-organization events reversed to regular phenotype and cells continued

productive growth at normal rates. As hypertonic re-organization of the nuclear periphery allows to interfere directly with the chromatin-lamina interaction in a dose-dependent manner, it appears to be a promising experimental tool to investigate the structural and functional integrity of the nuclear periphery in-vivo.

544/B495

Cell Cycle Mediated NAC-1 Cluster State and Nuclear Body Motility.

P. Wu[1], I. Shih[2,4], Y. Tseng[1,3]; [1]Chemical Engineering, University of Florida, Gainesville, FL, [2]Pathology and Oncology, Johns Hopkins Medical Institutions, Baltimore, MD, [3]Biomedical Engineering, University of Florida, Gainesville, FL, [4]Gynecology and Obsterics, Johns Hopkins Medical Institutions, Baltimore, MD

Nac-1 is a newly discovered protein that forms nuclear bodies within the nucleus, yet many of its functions are still unknown. Nac-1 is a member of the POZ/BTB gene family and a transcription repressor associated with cancer occurrence. Recently, using single particle tracking on stable cell line of Baby rat kidney (BRK) cell that is transfected with green-fluorescent protein (GFP) labeled Nac-1, we identified that the aggregation state and dynamics of the Nac-1 body are affected by the cell cycle. In the G1 stage, the Nac-1 nuclear body appears reduced in size and the mean squared displacement (MSD) reveals its movement to be more confined. Once the cell cycle progresses into the G2 stage, the Nac-1 nuclear body is augmented and it exhibits aggrandized motility. In addition, the nucleus staining results are shown to agree that Nac-1 aggregates have a spatial exclusion effect with chromatin. These experiments show that the activity of Nac-1 nuclear bodies is related to cell cycle, thereby suggesting the function and gene interaction abilities of Nac-1 nuclear bodies may be varied in each cell cycle stage.

545/B496

Multiscale Analysis of Nucleocytoplasmic Transport.

C. B. Wolf[1,2], M. Mofrad[1,2]; [1]Applied Science and Technology, UC Berkeley, Albany, CA, [2]Bioengineering, UC Berkeley, Berkeley, CA

The objective of this work is to further elucidate the connection between the biophysical and biochemical co-processes that occur during nucleocytoplasmic transport through the Nuclear Pore Complex (NPC) via a multiscale computational mechanical analysis. Currently, preliminary results indicate a novel connection between the biomechanical reconformations the NPC undergoes during transport events as well as the essential steps that are required for the efficient transport of a large variety of cargo complexes both in to and out of the nucleoplasm.The nuclear pore complex (NPC) is the only transitory pore for nucleocytoplasmic transport of a large variety of cargo complexes. Despite the actual biophysical and biochemical makeup, and even the size and weight, of the cargo complex, all the cargo complexes that are tagged with Importin-Beta and Importin-Alpha are localized for transport into the nucleoplasm. This type of transport behavior, being independent of the entire cargo complex sans the karyophilic co-factors of the Importins or the Exportins, suggests that the process of transport is one that combines a biochemical interaction with concerted biophysical changes in the overall state of the NPC during transport of both native cellular cargo complexes as well as xenogenic cargoes, such as viral nucleocapsids and pre-integration complexes. However, in the case of viral cargoes, it has been noted that they not only use a variety of techniques to import themselves into the nucleoplasm based on the particular strain of virus, but that not all of these techniques require usage or even biochemical mimicry of the native cellular karyophilic co-factors of Importin-Beta and Importin-Beta. Clearer understanding of the processes that regulate and modulate nucleocytoplasmic transport will be of great benefit towards the application of new clinical therapies and clinical techniques for continued research.

546/B497

Structural and Functional Domains of a Nucleolar Protein, NO66.

Y. Hirai, Y. Hirano, M. Kumeta, K. Takeyasu; Kyoto Univ., Laboratory of Plasma Membrane and Nuclear Signaling, Graduate School of Biostudies, Kyoto University, Kyoto, Japan

Nucleolar localization of specific proteins is critically regulated by several different levels; (i) some proteins require NLS (nuclear localization signal) and others do not, (ii) some proteins possess specific amino-acid sequences rich in lysine and arginine residues and others do not, and (iii) some proteins associate with co-factors such as B23 (nucleophosmin). In this study, we chose one of the nucleolar localizing proteins, NO66 (total amino acids 641), that is suspected to be a smallest protein with a histone or DNA-demethylation activity given by a JmjC domain (a.a.304-432), and dissected various domains responsible for nucleolar localization and association with other macromolecules. (1) NO66 possesses a putative NLS at its amino-terminal 49 amino acids (15PKRRRKPQPHSGSVLALPLRSRKIRK40) and thus a deletion mutant lacking this region (a.a.50-641) lost the nuclear localization. However, a.a.1-49 alone did not show the nucleolar localization. (2) NO66 localizes in the GC (Granular Component) region within the nucleolus during cell cycle except M-phase. A series of deletion mutations revealed that the amino-terminal region (a.a.50-303) was necessary for its nucleolar localization. (3) In comparison, Mina53, a homologue of NO66 did not contain such a distinct region, and any deletions abolished the nucleolar localization, and did give rise to nucleoplasm or cytosolic localizations. (4) Further amino-acid sequence comparisons among JmjC-containing proteins and deletion mutagenesis indicated that there was no distinct amino-acid sequence that specifically determined the nucleolar localization. Thus, we concluded that tertiary structures, but not particular amino-acid sequences themselves, are important for the nucleolar localization. (5) NO66 has been known to associate with B23. In addition to this protein-protein interaction, NO66 also bound to DNA at its amino-terminal region (a.a.1-49). In summary, it seems that NO66 is transported into the nucleus via its NLS, localized to the nucleolus with a help of other molecules, and interact with DNA.

Developmental Control of Gene Expression (547-557)

547/B498

The Role of the Nuclear Periphery in Mouse Embryonic Stem Cell Differentiation.

C. H. Pratt, L. S. Shopland; The Jackson Laboratory, Bar Harbor, ME

The location of genes within the nucleus can impact gene expression, but the extent to which gene activity is related to their nuclear compartmentalization is poorly understood. One nuclear compartment, the nuclear periphery, is thought to contain silenced genes that relocate to the interior of the nucleus when activated. However, recent data indicate that a sub-set of genes in gene-poor regions of chromatin remain at the nuclear periphery when active, suggesting that the nuclear periphery may play a role in the expression of at least some genes. In embryonic stem (ES) cells, a large contingent of genes poised for activation are marked by a unique bivalent epigenetic signature, whereby the histones surrounding the gene display both an active (H3K4Me3) and inactive (H3K27Me3) methylation pattern. We tested whether bivalent genes repressed in ES cells change their nuclear location relative to the nuclear periphery upon differentiation to primitive mesoderm. Using the mouse ES cell line, E14, we identified, by computational, microarray and qRT-PCR analysis, a group of 60 genes that reside in areas of low gene density throughout the mouse genome, are significantly up-regulated during ES cell differentiation to primitive mesoderm and are epigenetically bivalent. Homogenous populations of either mesodermal or ES cells were obtained by Flow Cytometric sorting of cells into FLK-1 or SSEA-1 positive cell populations, respectively. Fluorescent in situ Hybridization (FISH) was used to determine whether the selected genes localize to either the nuclear periphery or nuclear center in both undifferentiated ES and primitive mesoderm cells. We find that a sub-set of genes residing in areas of low gene density remain at the nuclear periphery when active during mouse ES cell differentiation, suggesting that the nuclear periphery may play an active role in the regulation of poised genes during mouse embryogenesis.

548/B499

Epigenetic Regulation of Gene Expression in the Chicken by Neonatal Programming.

C. M. Ashwell; North Carolina State University, Raleigh, NC

Epigenetic regulation of gene expression was initially described as genomic imprinting where a gene is expressed in a parent-of-origin-dependent manner. Another form of epigenetic regulation has been recently described called fetal “programming”. Fueled by epidemiological data the “fetal origins” hypothesis suggests that a poor in utero environment resulting from maternal dietary or placental insufficiency may “program” susceptibility in the fetus to cardiovascular or metabolic disease. Recent evidence indicates this process is through genome modification by methylation of CpG islands and modification of gene expression. In birds, the first description of neonatal programming was referred to as early-life conditioning and was manifest as heat tolerance late in life as a result of neonatal thermal conditioning. We have observed similar apparent programming by dietary manipulation. When chickens are challenged with a diet low in phosphorus (P) for 90 hours post-hatch they obtain the ability to better utilize P later in life. This increased retention of P from the diet can partially be explained by an increase in the expression of the intestine-specific Na/P cotransporter (NaPcoT) gene during programming as well as later in life when fed P restricted diets. Upon investigation of CpG islands upstream of the NaPcoT gene a pattern of reduced methylation is observed as a result of early P restriction. Microarray analysis demonstrates that the expression of many genes appear to be regulated by dietary P in the intestine. This is the first evidence for neonatal programming of gene expression in an oviparous species as well and provides essential information on the mechanism of nutritional programming in chickens. Feed is the most expensive component of poultry production and any fractional improvement in nutrient utilization will result in a significant economic impact. Reduction in manure nutrients such as P will also significantly decrease the impact of poultry production on the environment.

549/B500

Expression of Chromatin Remodeling Factors PRDM10 during Mouse Embryonic Development.

K. Kim; Division of Life Sciences, College of Natural Sciences, Kangwon National University, Chuncheon, South Korea

During the early vertebrate development, the tissue or organ -specific genes control cell fate by genetic developmental program. Recently, many concerns have been increased on chromatin remodeling factors regulating histone modification. PRDM10, PR domain containing zinc finger proteins, may play essential role for chromatin structural changes of chromatin. In this study, we performed immunohistochemistry in various developmental stages after mating with 8 weeks old ICR mouse. Embryos were obtained from E9.5, 11.5, 16.5 and postnatal stages. During gastrulation (E9.5), PRDM10 is mainly expressed in mesenchymal cells in mesoderm. At E11.5 that organ formation begins, PRDM10 are strongly expressed in somites, neural ganglian, face and notochord. The expression of face, chondrocyte, skeletal muscle, bladder, dermis and heart derived from mesenchymal tissues is also shown at fetal maturation stage (E16.5). In contrast, PRDM10 is expressed various tissues derived from endoderm, mesoderm, and ectoderm at postnatal stage. With the present study, we suggest that PRDM10 is a central role in mesoderm differentiation during mouse development.

550/B501

A Role for Basic Transcription Element Binding Protein 1 (BTEB1) in Regulation of the Fibroblast Growth Factor Receptor 1 (FGFR1) Gene during Skeletal Myogenesis.

D. Mitchell, J. Dimario; Cell Biology and Anatomy, Rosalind Franklin University of Medicine and Science, North Chicago, IL

Regulation of chicken skeletal myogenesis is mediated in part by transcriptional regulation of the fibroblast growth factor receptor 1 gene (FGFR1). Proliferating myoblasts express the FGFR1 gene which is positively regulated by Sp1. FGFR1 gene expression is down-regulated in differentiated myotubes and negatively regulated by E2F4. Since transcriptional regulation of gene expression is typically a process that involves many transcription factors working in concert, it is likely that other transcription factors are important in the regulation of FGFR1 gene expression during myogenesis. Basic transcription element binding protein 1 (BTEB1) is one of those factors, and it has been reported to positively and negatively regulate promoter activity of various genes. Our goal is to identify its effect on FGFR1 gene expression during myogenic cell proliferation and differentiation. Reverse-transcriptase PCR (RT-PCR), immunocytochemistry, and western blot experiments determined the presence of BTEB1 in proliferating myoblasts as well as differentiated myotubes. Transient cell transfection assays using plasmid constructs harboring the full-length wild-type chicken FGFR1 gene promoter linked to the luciferase reporter gene, demonstrated dose-dependent transcriptional activation by BTEB1. Additional transcription reporter experiments with constructs carrying mutations of

FGFR1 cis-elements showed decreased luciferase activity, suggesting interaction of BTEB1 with the FGFR1 promoter. Preliminary chromatin immunoprecipitation assays confirmed that BTEB1 transactivates the FGFR1 promoter by binding directly to the DNA. Together these results show that BTEB1 helps coordinate the expression of the FGFR1 gene during myogenic cell proliferation and differentiation. These results may also help increase fundamental knowledge about cell proliferation in general.

551/B502

Characterization of the Holozoan Nucleolar Ribosome Biogenesis Compartment.

A. J. Erives[1], S. Brown[2], M. Cole[3,2]; [1]Dept. of Biological Sciences, Dartmouth College, Hanover, NH, [2]Dept. of Genetics, Dartmouth Medical School, Lebanon, NH, [3]Dept. of Pharmacology and Toxicology, Dartmouth Medical School, Lebanon, NH

The objective of this study is to use comparative regulatory genomics to characterize the set of genes controlling ribosome biogenesis in metazoan systems. The ribosome biogenesis (RiBi) genes encode a highly-conserved eukaryotic set of nucleolar proteins and non-coding RNAs involved in rRNA transcription, assembly, processing, and export from the nucleus. Here we show the existence of a definitive regulatory signature for the entire RiBi regulon in holozoan genomes. This distinctive signature allowed us to identify the entire RiBi gene set in the genome of Drosophila melanogaster. We then used multiple genome-wide analyses to show that this distinctive mode of RiBi regulation co-evolved with the Myc and Max genes in a stem-holozoan, the ancestor of both Metazoa and Choanoflagellata, which is the sister group to animals. Additionally, we have implicated several new genes as belonging to this fascinating and complex cellular compartment. Most of these genes are distinct from ribosomal proteins. These results indicate that a Myc regulon, which is activated in proliferating cells during normal development as well as during tumor progression, has primordial roots in the evolution of an inducible growth regime in a protozoan ancestor of animals. Furthermore, by comparing divergent basic Helix-Loop-Helix (bHLH) repertoires, we conclude that regulation by Myc but not by other bHLH genes is responsible for the evolutionary maintenance of Myc binding sites across the RiBi suite of genes. This work will lay the foundation for a sytematic study of nucleolar function in metazoan cells.

552/B503

Pitx3 Potentiates Nurr1 in Dopamine Neuron Terminal Differentiation through Release of SMRT-mediated Repression.

F. M. Jacobs, S. van Erp, A. J. Van der Linden, L. von Oerthel, J. H. Burbach, M. P. Smidt; Rudolf Magnus Institute of Neuroscience, Utrecht, Netherlands

In recent years, the meso-diencephalic dopaminergic (mdDA) neurons have been extensively studied for their association with Parkinson's disease. Thus-far, specification of the dopaminergic phenotype of mdDA neurons is largely attributed to the orphan nuclear receptor Nurr1. In this study, we provide evidence for extensive interplay between Nurr1 and the homeobox transcription factor Pitx3. We demonstrate the shared interaction of Pitx3 and Nurr1 with the co-repressor PSF and identify the occupancy of Nurr1 target gene promoters by both Pitx3 and Nurr1. Moreover, in vivo expression analysis reveals that Nurr1 by itself is not sufficient to drive the dopaminergic phenotype and requires Pitx3 for full activation of target gene expression. In the absence of Pitx3, Nurr1 is kept in a repressed state through the interaction with the co-repressor SMRT. Highly resembling the effect of ligand activation of nuclear receptors, recruitment of Pitx3 modulates the Nurr1 transcriptional complex by decreasing the interaction with SMRT and releasing the HDAC mediated repression of Nurr1 target genes. These data position Pitx3 next to Nurr1 in specifying the dopaminergic phenotype, providing novel insights into mechanisms underlying development of mdDA neurons in vivo, and programming of ES-cells as future cell replacement therapy for Parkinson’s disease.

553/B504

Nuclear Actin Is a Key and Early Player for Laminin-induced Transcriptional Repression and Growth Inhibition in the Mammary Gland.

V. Spencer[1], S. Costes[1], J. Inman[1], J. Chen[1], R. Xu[1], M. J. Hendzel[2], M. Bissell[1]; [1]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, [2]Department of Oncology, Cross Cancer Research Institute, Edmonton, AB, Canada

Beta-actin is now accepted to play an important role in many events related to gene expression. The microenvironment in general and the extracellular matrix, in particular, are known to affect many tissue-specific functions and alter gene expression. Whether ECM molecules influence the levels and function of nuclear beta-actin is unknown. Here, we show that beta-actin levels decreased by more than 50% within the nucleus and cytoplasm of mammary epithelial cells shortly after treatment with laminin 111 (LN 111), an inducer of differentiation for this cell type. This decrease preceded cell cycle arrest and coincided with a decrease in RNA-polymerase II activity. Other ECM molecules tested did not significantly influence beta-actin levels. Over-expression of a NLS-beta-actin construct was sufficient to partially reverse LN 111-mediated transcriptional repression. Immunostaining analysis of the mammary epithelium in the terminal end bud of a developing mouse mammary gland demonstrated an inverse correlation between high levels of transcriptional activity and the presence of an intact LN 111-containing basement membrane. Thus, nuclear beta-actin serves as a target through which the microenvironment invokes changes in RNA polymerase II-dependent events that likely contribute towards cell cycle arrest and the onset of epithelial cell functional differentiation in the developing mammary gland.

554/B505

Analysis of the Molecular Mechanism Underlying the Neural RNA-binding Protein Musashi1-mediated Translational Repression.

H. Kawahara[1,2], T. Imai[1,2], H. Imataka[3], M. Tujimoto[4], K. Matsumoto[4], H. Okano[1,2]; [1]Physiology, Keio University School of Med., Tokyo, Japan, [2]SORST, JST, Saitama, Japan, [3]RIKEN, Yokohama, Japan, [4]RIKEN, Cellular Biochemistry, Wako, Japan

Musashi1 (Msi1) is an RNA-binding protein that is highly expressed in neural stem cells. We previously reported that Msi1 contributes to the maintenance of the immature state and self-renewal activity of neural stem cells through the translational repression of *m-Numb*. However, its translation repression mechanism has remained unclear. Here, we identified PABP, IMP, and so on as Msi1-binding protein, and found Msi1 competes with eIF4G for PABP binding. This competition inhibited translation initiation of Msi1's target mRNA. Indeed, deletion of the PABP-interacting domain in Msi1 abolished its function. We demonstrated that Msi1 inhibited the assembly of the 80S, but not the 48S ribosome complex. Consistent with these conclusions, Msi1 colocalized with PABP and was recruited into stress granules, which contain

stalled preinitiation complex. These results provide insight into the mechanism by which sequence-specific translational repression occurs in stem cells through the control of translation initiation. Moreover we address Msi1 complex, particularly relation among Msi1, IMP3 and Lin28. IMP3 and Lin28 proteins are also expressed in neural tube. Lin28 is known to regulate the neural stem-cell commitment via microRNA biogenesis. It is coincident that Msi1 localized in cytoplasmic foci (P-bodies). Taken together, we will discuss in relation to these Msi1-complex.

555/B506

Regulation of c-myb by NF-κB Family Members during Murine Erythroleukemia Differentiation.

J. L. Kowal, A. Lelis, C. Toth; Biology, Providence College, Providence, RI

c-Myb is a transcription factor and proto-oncogene that is important for hematopoietic cell development. The regulation of the c-Myb protein and its down stream targets is well studied while the regulation of expression of the *c-myb* gene is not well understood. *c-myb* expression is controlled by a conditional block in transcriptional elongation in the first intron of the gene. Previous studies have shown that NF-κB family members are involved in regulating the activation of *c-myb* by binding to Rel-Related Proteins Binding Elements (RRBE) in the first intron. This leads to the hypothesis that NF-κB regulates expression of *c-myb* during development of hematopoietic cells. Here we show using chromatin immunoprecipitations (ChIPs) of murine erythroleukemia cells that a block in pre-mature transcription arrest caused by hexamethylene bisacetamide (HMBA)-induced differentiation can be over-come by stimulation of adenyl cyclase by forskolin. RNA polymerase II density studies demonstrate that polymerase II levels decrease in exon 2 following HMBA treatment. Co-treatment with forskolin results in polymerase II density in exon 2 returning to levels observed in untreated cells. ChIP analysis of NF-κB occupancy of the two RRBE elements in the first intron show that p50 and RelB bind to the 5' RRBE site exclusively. This study demonstrates for the first time a correlation between NF-κB occupancy of RRBE sites and *c-myb* expression in vivo in hematopoietic cells.

556/B507

Proteomic Response of the Sea Urchin, Strongylocentrotus purpuratus, Early Cleavage Embryo to Ultraviolet Radiation.

J. P. Campanale, L. Tomanek, N. Adams; Biological Sciences, California Polytechnic State University, San Luis Obispo, San Luis Obispo, CA

Increasing solar ultraviolet radiation (UVR, 290-400 nm), especially ultraviolet B, (UVB, 290-320 nm) is reaching Earth's surface due to ozone depletion and global climate change. Embryos of the purple sea urchin, *Strongylocentrotus purpuratus*, provide an ideal system for modeling the protein-mediated cell cycle response to stressful UV-irradiation. Six batches of *S. purpuratus* embryos were exposed to UVR and monitored for delays in mitotic cleavage. Protein expression profiles from UV-treated and UV-protected embryos were obtained using two-dimensional gel electrophoresis (2D GE). Eggs were fertilized, exposed to UVR using a Q-Panel UV-340 lamp and allowed to develop for 30 and 90 mins. Subsequently, proteins were isoelectrically focused (pH 4-7) and separated by molecular weight using SDS-PAGE. UV-treated embryos cleaved an average of 21.69 mins later than the UV-protected embryos. At least 987 protein spots were detected in the gels containing either UV-protected or UV-treated cell lysates. Our preliminary results indicate that 141 protein spots show a significant change in expression density between UV-treatments for all batches of embryos at all time points (2-way ANOVA, P= 0.01). We are evaluating these protein spots for promise as markers of UV-induced stress by identification using matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF-TOF) mass spectrometry. Our findings indicate that protein expression profiles obtained using 2D GE and MALDI-TOF mass spectroscopy are valuable tools for identifying how embryos respond to UVR -irradiation. Identification of these proteins may allow us to further ascribe functional stress responses at the cellular level.

557/B508

HIF-1α-dependent Expression of Nkx2.5 Is Required on Normal Heart Development in Xenopus.

K. Nagao[1,2], Y. Taniyama[2], N. Mochizuki[1], R. Morishita[2]; [1]Structural Analysis, National Cardio Vascular Center, Osaka, Japan, [2]Clinical Gene Therapy, Osaka University, Osaka, Japan

[Background] Hypoxia inducible factor-1α (HIF-1α) is originally identified as a transcription factor that activates genes expression in response to hypoxia. HIF-1α functions as a master regulator of oxygen homeostasis and regulates adaptive responses to change in oxygen tension during embryogenesis, tissue ischemia, and tumorigenesis. Analysis of HIF-1α knockout mice, which are embryonic lethal due to a defect in vasculogenesis, revealed that HIF-1α signaling is essential for proper vascular development. Since HIF-1α-deficient mice exhibit a number of developmental defects, the precise role of HIF-1α in early cardiac morphogenesis has been uncertain. Therefore, to clarify the role of HIF-1α in heart development, we investigated the effect of knockdown of HIF-1α in Xenopus embryos using antisense morpholino oligonucleotide microinjection techniques. [Methods and Results] The effect of decreased expression of HIF-1α on embryogenesis was performed with using antisense morpholino oligonucleotide-mediated knockdown of HIF-1α. The hif-1α antisense morpholino injected into one of the two vegetal-dorsal blastomeres at the 8-cell stage of Xenopus embryos. Then we performed whole-mount in situ hybridization for tnIc that revealed the knockdown of HIF-1α caused cardia bifida at stage 32 embryos. These defects may result from abnormal cardiomyocyte differentiation. To analyze the molecular basis of the defect in heart development of HIF-1α-depleted embryos, we examined the expression of five transcriptional factors (nkx2.5, gata4, tbx5, hand1, hand2) and one inductive molecule (bmp). It was found that reduced expression of nkx2.5 was observed with the knockdown of HIF-1α. Luciferase reporter assay using the nkx2.5 promoter showed that the knockdown of HIF-1α decreased its promoter activity. Furthermore, overexpression of Nkx2.5 could rescue the cardiac abnormality in Hif-1α morpholino injected embryos. [Conclusions] The present study would provide new information that regulation of nkx2.5 by HIF-1α is necessary for normal cardiac development. HIF-1α is needed to form proper heart morphology besides vascular development.

Epithelia I (558-571)

558/B510

Exfoliation of Gastric Pit-parietal Cells into the Gastric Lumen Associated with a Stimulation of Isolated Rat Gastric Mucosa in Vitro: A Morphological Study by the Application of Cryotechniques.

F. Aoyama, A. Sawaguchi, S. Ide, T. Suganuma; Anatomy, University of Miyazaki, Faculty of Medicine, Miyazaki, Japan

It is clinicopathologically important to elucidate the cell kinetics for the maintenance of normal gastric epithelium. In a rat gastric mucosa isolated after stimulation, a number of cells were exfoliated into the gastric lumen of the pit region. The present study was undertaken to clarify the origin of exfoliated cells and their histochemical profiles by taking the advantages of cryotechniques. As results, most of the exfoliated cells were identified as pit-parietal cells labeled with both peanut-lectin and anti-H+/K+-ATPase antibody. Quantitative analysis verified a time-dependent increase in the number of exfoliated cells in the gastric mucosa isolated after stimulation. The exfoliated cells exhibited a diffuse intracellular staining for E-cadherin, suggesting a dissociation of the adhesion molecule prior to the cell exfoliation. It should be noted that most of the exfoliated cells were negative to the apoptotic markers (TUNEL staining and caspase-3). Ultrastructurally, autophagosome-like structures consisting of H+/K+-ATPase positive membranes were frequently seen in the exfoliated pit-parietal cells. In addition, the pit-parietal cell exfoliation was accompanied by sealing of their basal portion with the cytoplasmic processes of adjacent surface mucous cells. The present morphological findings provide a new insight into the cell kinetics in the gastric epithelium in vitro.

559/B511

Establishment of In Vitro Model for Murine Intestinal M Cell Differentiation.

T. Kanaya[1], K. Miyazawa[2], I. Takakura[1], W. Itani[1], H. Watanabe[1], T. Hondo[1], K. Watanabe[1], S. Ohwada[1], T. Yamaguchi[1], H. Aso[1]; [1]Tohoku University, Sendai, Japan, [2]Yale Medical School, New Haven, CT

M cells are a kind of intestinal epithelial cell in the follicle-associated epithelium (FAE) of Peyer's patches (PP). They can transport antigens and microorganisms from gut lumen to underlying lymphoid tissues. Despite the important role of M cells in mucosal immune responses, the origin and differentiation of M cells as well as the cell death remain unclear. In order to clarify the mechanism of M cell differentiation, we established a novel murine intestinal epithelial cell line (MIE) from the C57BL/6 mouse. MIE cells grew rapidly and had a cobblestone morphology, which was a typical feature of intestinal epithelial cells. Additionally, they expressed cytokeratin, villin, cell-cell junctional proteins and alkaline phosphatase activity, and formed microvilli. The expression of Musashi-1 antigen indicates that MIE cells may be close to intestinal stem cells or transit-amplifying cells. MIE cells differentiated into the M cell lineage following co-culture with intestinal lymphocytes, but not with Peyer's patch lymphocytes (PPL). PPL co-stimulated with anti-CD3/CD28 monoclonal antibodies (mAbs) caused MIE cells to display typical features of M cells, such as transcytosis activity, the disorganization of microvilli and the expression of M cell markers (annexin V and sgne-1). In order to determine which cell types in PPL are essential for the induction of M cell differentiation of MIE cells, we co-cultured MIE cells with T and B cells isolated from PPL. The transcytosis activity of MIE cells was not induced by T cells co-stimulated with the mAbs, and was reduced by the depletion of the T cell population from PPL. A mixture of T cells treated with mAbs and B cells led MIE cells to differentiate into M cells. We report here that MIE cells have the potential ability to differentiate into M cells, and that the differentiation requires activated T cells and B cells.

560/B512

MicroRNA-98 and let-7 Regulate Expression of Cytokine-induced SH2 Protein and Modulate Microbial-stimulated NF-kappaB Activation in Epithelial Cells.

G. Hu, R. Zhou, A. Gong, X. Chen; Med Microbiol Immunol, Creighton University, Omaha, NE

The signaling pathways associated with Toll-like receptors (TLRs) and nuclear factor-kappa B (NF-kappaB) not only mediate epithelial immunity but are also important to adaptive immune responses to microbial infection. A fine-turning of the TLR signaling cascades in epithelial cells is key to maintaining of mucosal homeostasis. The cytokine-inducible Src homology 2 protein and suppressors of cytokine signaling (CIS/SOCS) are a family of intracellular proteins and have emerged as key physiological regulators of inflammatory cytokine responses. Here, we report that CIS is the target for miR-98 and let-7. Binding of miR-98 or let-7 to the CIS 3'-UTR results in translational repression of CIS in cultured human biliary epithelial cells (cholangiocytes). Activation of the TLR signals by lipopolysaccharide stimulation or infection of Cryptosporidium parvum, a parasite that activates TLR2 and TLR4, increases CIS protein expression in cholangiocytes. Importantly, LPS stimulation or C. parvum infection decreases expression of both miR-98 and let-7. Induced suppression or forced expression of miR-98 and let-7 causes reciprocal alterations in lipopolysaccharide- and C. parvum-stimulated CIS protein expression. Moreover, expression of CIS protein is associated with an accelerated degradation of IkappaB-alpha and thus feedback regulates activation of the NF-kappaB pathway. The data support a miRNA-mediated feedback regulation of NF-kappaB signaling via modification of CIS expression in cholangiocytes, a process that may be relevant to regulation of TLR/NF-kappaB signaling in general.

561/B513

Novel Discoveries about Architecture of Gut Epithelial Tissue with Implications for the Early Stages of Colorectal Cancer and the Role of the Adenomatous Polyposis Coli Protein.

S. Nelson, P. Appleton, I. Nathke; University of Dundee, Dundee, United Kingdom

Colorectal cancer (CRC) is a leading cause of cancer death worldwide. Consequently, the molecular basis of CRC has been examined closely. A feature common to most CRCs is mutation of the Adenomatous polyposis coli (APC) gene. Loss of APC function in mouse models is sufficient for tumorigenesis. The APC protein regulates Wnt signaling, actin, and microtubules (MTs). Carcinogenic truncations in APC lead to a loss of both actin and MT interacting domains and changes in Wnt signalling, suggesting that all these functions of APC are relevant to CRC. Indeed, cell biological evidence implicates the APC protein in all the processes that govern normal gut tissue architecture: migration, differentiation, proliferation and apoptosis. In this study, we aimed to determine how changes that are induced by mutations in APC in simpler experimental systems correlate with changes in the relevant tissue. To this end we compared the architecture of mouse gut with or without mutant APC using multi-photon microscopy. We established quantitative parameters that describe this tissue in three dimensions and then used these parameters to identify the earliest measurable architectural changes in APC mutant tissue, which appears

normal by conventional histological analysis but gives rise to tumours early in life. We found that in both, large and small intestine, crypts are less regular in size and shape in mice heterozygous for Apc (a well established model for familial adenomatous polyposis patients). Crypt fission is altered, cell size is more variable and mitotic spindles may be oriented differently. Our data provide important validation of data acquired in less complex experimental systems but also reveal completely new insights into the organisation of intestinal tissue. We recently expanded this technique to enable time-lapse analysis of intact, live gut tissue. Using the parameters obtained from fixed tissue we validated the viability of the living tissue during recording times of 3-4 days. We compare fluorescence labelling methods for live imaging. In the future, we will use live image analysis to answer questions about basic gut physiology and the first stages of tumourigenesis.

562/B514

Enhanced Colon Cancer Cell Survival by Thrombospondin-1 from Sulindac Sulfide-mediated Apoptosis.

H. Yang, Y. Moon; Department of Microbiology and Immunology, Pusan National University School of Medicine, Busan, South Korea

Previously, we reported that non-steroidal anti-inflammatory drugs (NSAIDs) suppress tumor cell invasion which was mediated by thrombospondin-1 (TSP-1). As the extending study of the previous observation, we investigated the effect of NSAID-induced TSP-1 on the cancer cellular survival and its related signaling transduction of the TSP-1 production. Among diverse NSAIDs, sulindac sulfide was most potent of inducing the human TSP-1 protein expression. Functionally, induced TSP-1 expression was associated with the growth-compensatory action of NSAID. TSP-1 expression was also elevated by mitogenic signals of ERK1/2 and RhoA GTPase pathway which had also growth-promotive capability after sulindac sulfide treatment. These findings suggest the possible mechanism through which tumor cells can survive the chemo-preventive action of NSAIDs in a compensatory way. This study will provide an insight into the detail safety issues of NSAID as the anti-cancer agents in the patient in the future study.

563/B515

TGF-β1-induced Upregulation of β1 Integrin in Epithelial-to-Mesenchymal Transition.

Y. Yeh, W. Wei, Y. Wang, H. Lin, M. Tang; Basic Medical Sciences, National Cheng-Kung University, Tainan, Taiwan

TGF-β1-induced epithelial-to-mesenchymal transition (EMT) contributes to pathophysiological mechanism in kidney fibrosis during chronic renal failure. However, the molecular mechanisms whereby TGF-β1 induced EMT have not been fully understood. In this study, we first examined whether β1 integrin signal is required for TGF-β1-induced EMT in LLC-PK1 cells. TGF-β1 triggered decrease in E-cadherin and elevation of EMT marker proteins, such as α-SMA and fibronectin within 24h. However, TGF-β1 induced β1 integrin induction within 8h, which could be completely inhibited by actinomycin D. Both blocking and knockdown of β1-integrin signaling significantly inhibited TGF-β1-induced EMT. TGF-β1 stimulation induced Smad2/3 activation and knockdown of Smad3 protein levels prevented TGF-β1-induced β1 integrin expression. Bioinformatics search predicted a Smad3 and Smad4 binding element on β1 integrin promoter. We found that TGF-β1 triggered β1 integrin promoter activity in a dose dependent manner. Both knockdown Smad3 and mutation of Smad-binding element (SBE) significantly blocked TGF-β1 induced β1 integrin promoter activity. Results of chromatin immunoprecipitation (ChIP) assay showed that Smad3 direct binds on β1 integrin promoter region upon TGF-β1 stimulation. In order to examine the change of β1 integrin in EMT during kidney fibrosis, we employed unilateral ureter obstruction (UUO) rat model. We found that the β1 integrin levels were increased, accompanied with the upregulation and activation of Smad2/3 within 3d of UUO. Immunohistochemical studies showed that β1 integrin was colocalized with α-SMA staining in tubule epithelial cells in UUO kidney. Taken together, TGF-β1 upregulates gene expression of β1 integrin, which plays an important roles in TGF-β1-induced EMT both in vitro and in vivo.

564/B516

Podocalyxin and NHERF1 Recruit Regulators of Apical Membrane to Promote Single Lumen Formation.

A. Datta, D. Bryant, K. Mostov; Anatomy, UCSF, San Francisco, CA

Epithelial organs in vivo share in common a polarized architecture, comprising of a central lumen surrounded by a layer of cells. The molecular composition of the apical plasma membrane is critical to the formation and maintenance of this polarized state. We have used a 3-dimensional cell culture system to study the early determinants of single lumen formation and maintenance of apical phospholipid composition. Podocalyxin (PCX, also known as gp135) is one of the earliest proteins to localize to the nascent apical lumen. ShRNA-mediated silencing of PCX inhibits single lumen formation, underscoring its essential role on this process. NHERF1 and PCX associate with each other and co-distribute during cyst development. A mutant of NHERF1, unable to bind the Ezrin/Radixin/Moesin (ERM) family of actin-binding proteins, also disrupts single lumen formation, suggesting that NHERF1 recruits the ERM family of proteins to the luminal membrane, which in turn stabilizes the apical actin cytoskeleton. Phosphatidylinositol-4-phosphate 5 kinase 1-β (PI4P5K1-β), another binding partner of NHERF1, also localizes to the luminal boundary. A mutant of PI4P5K1-β, defective in NHERF1 binding, disrupts single lumen formation and fails to localize to the luminal boundary, indicating that NHERF1 at the apical membrane is required for recruiting PI4P5K1-β, which functions to shift the phospholipid equilibrium towards PI(4,5)P2. Our findings suggest that PCX association with NHERF1 at the lumen serves to recruit the regulators of two essential elements of apical identity - actin polymerization and phospholipid composition.

565/B517

MicroRNA-513 Regulates B7-H1 Translation and Is Involved in Interferon-gamma-induced B7-H1 Expression in Cholangiocytes.

A. Gong[1], R. Zhou[1], G. Hu[1], X. Li[1], P. Splinter[2], S. O'Hara[2], N. LaRusso[2], G. A. Soukup[3], H. Dong[4], X. Chen[1]; [1]Med Microbiol Immunol, Creighton University, Omaha, NE, [2]Gastroenterology and Hepatology, Mayo Clinic College of Medicine, Rochester, MN, [3]Biomedical Science, Creighton University, Omaha, NE, [4]Department of Immunology, Mayo Clinic College of Medicine, Rochester, MN

Biliary epithelial cells (cholangiocytes) respond to pro-inflammatory cytokines, such as interferon-gamma (IFN-gamma), and actively participate in the regulation of biliary inflammatory response in the liver. B7-H1 (CD274, PD-L1) is a member of the B7 costimulatory molecules and plays a critical immunoregulatory role in cell-mediated immune responses. Here, we show that resting human cholangiocytes express B7-H1 mRNA, but not B7-H1 protein. IFN-gamma induces B7-H1 protein expression and alters miRNA expression profile in

cholangiocytes. Of those IFN-gamma-downregulated miRNAs, we identified miR-513 with complementarity to the 3'-UTR of B7-H1. Targeting of miR-513 to B7-H1 mRNA results in translational repression, but not B7-H1 mRNA degradation. Transfection of cholangiocytes with an antisense oligonucleotide to miR-513 induces B7-H1 protein expression. Additionally, overexpression of miR-513 precursor decreases IFN-gamma-induced B7-H1 protein expression and consequently, interferes with associated apoptotic cell death in co-cultured Jurkat cells. Thus, miR-513 regulates B7-H1 translation and mediates IFN-gamma-induced B7-H1 expression in human cholangiocytes, suggesting a role for miRNA-mediated gene silencing in the regulation of cholangiocyte response to IFN-gamma.

566/B518

Alterations in Oxidative Stress, Apoptotic Pathways, Calcium Metabolism and Gene Expression in Human Bronchial Epithelial Cells by Zinc Oxide Nanoparticles.

C. Huang[1], D. Chen[2], R. S. Aronstam[1], Y. Huang[1]; [1]Biological Sciences, Missouri University of Science and Technology, Rolla, MO, [2]Energy, Environmental and Chemical Engineering, Washington University in St. Louis, St. Louis, MO

The influences of 20 nm zinc oxide nanoparticles on intracellular calcium levels and the expression of genes involved in apoptosis and cellular responses to oxidative stress were studied in human bronchial epithelial cells (BEAS-2B). Exposure to zinc oxide particle suspension caused a dose- and time-dependent cytotoxicity. This toxicity was observed at ZnO concentrations as low as 5-6 µg/mL; there was a steep decline in cell numbers at concentration between 6-10 µg/mL. ZnO increased the levels of intracellular reactive oxygen species levels as well as the release of lactate dehydrogenase into the cell culture media, in a dose-dependent manner. Treatment with an antioxidant, N-acetylcysteine (NAC), prevented cell loss, indicating oxidative stress mediated cytotoxicity. Exposure to ZnO significantly increased intracellular calcium levels in a dose- and time-dependent manner. Intracellular calcium concentrations (measured using imaging of fura-2 fluorescence) were elevated by as much as four fold compared to control cells, and this elevation was partially attenuated by NAC. The calcium channel blockers verapamil and nifedipine did not affect the elevation intracellular calcium. The source(s) of excess calcium in the cytosol is unclear. Exposure to a subtoxic dose of ZnO nanoparticles (5 µg/mL) increased the expression of BNIP, PRDX3, PRNP, and TXRND1, genes which are involved in apoptosis and oxidative stress responses. The expression of these genes increases by at least 2.5 fold. Thus, exposure of BEAS-2B cells to 20 nm ZnO particles results in 1) a dose- and time- dependent cytotoxicity reflected in cell viability reduction, elevated oxidative stress, and cell membrane damage; 2) a dose-dependent elevation of intracellular calcium levels, and 3) up-regulation of several genes induced in apoptosis and oxidative stress responses. The relationships between the altered expression redox sensitive genes and the elevated intracellular calcium levels remain to be elucidated. Supported by the MS&T cDNA Resource Center (www.cdna.org).

567/B519

Well-differentiated Respiratory Epithelial Cells of Bovine Airways Show Different Sensitivity to Bovine Respiratory Viruses.

K. Goris, S. Uhlenbruck, C. Schwegmann-Webels, G. Herrler; Institute of Virology, University of Veterinary Medicine Hanover, Hannover, Germany

Investigations of the pathogenesis and morphological alterations of infections by respiratory viruses often depended on *in vivo* models, which in the case of bovine viruses hold difficulties. To investigate different viral strategies of host infection *in vitro*, we characterized two models for primary cultures of differentiated bovine bronchial epithelial cells (BAEC): (a) Under air-liquid-interface (ALI) conditions, BAEC differentiate into a pseudo-stratified epithelium being characterized by the presence of ciliated cells and goblet cells after 2-3 weeks. (b) Precision-cut lung slices (PCLS), obtained from bovine lungs, enable infection studies with BAEC in the original tissue organisation. The PCLS are suitable for experiments up to seven days after preparation, as determined by different criteria of vitality, like cilia activity and bronchoconstriction. Our analysis focused on *bovine respiratory syncytial virus* (BRSV) and *bovine para-influenza virus type 3* (BPIV3), known as the major causes of respiratory disorders in calves. We found that BPIV3 preferentially infects ciliated cells of the respiratory epithelium, as has been reported for human PIV3 (Zhang *et al*., 2005). By contrast, infection of the respiratory epithelium by BRSV is restricted to few non-ciliated cells, which appear to be less differentiated. In PCLS, infection by BRSV occurs only in subepithelial tissues. We conclude that this approach provides two adequate *in vitro* models for further investigations of the sensitivity of respiratory epithelial cells to infections by bovine respiratory viruses.

568/B520

Effect of Inhaled Soot on Neonatal and Adult Rats.

L. S. Van Winkle[1,2], P. C. Edwards[1], J. A. Mathison[1], K. M. Sutherland[1], J. K. Chan[1], V. L. Mitchell[1], D. S. Anderson[1]; [1]Center for Health and the Environment, UC Davis, Davis, CA, [2]School of Vet Med: Anatomy, Physiology and Cell Biology, UC Davis, Davis, CA

Exposure to unhealthy levels of particulate air pollution (PM) is widespread and has been correlated with increased morbidity and mortality in adults and with an increased incidence of asthma in children. PM is composed of more than just particulates; it contains allergens, metals, soil and combustion products including organic compounds bound to the particulates as well as in the gas phase. As a first step towards defining which components of PM are important, we exposed neonatal (7d old) and adult male Sprague Dawley rats to inhaled soot generated by a diffusion flame for 6 hrs. Control rats were exposed to filtered air. This soot was low in organic carbon and did not contain many of the other elements associated with ambient PM; thus it simulated the particulate fraction of ambient PM. Airway epithelial cell injury was assessed 2 hrs post exposure using differential permeability to fluorescent dyes and high resolution histopathology and by measuring LDH release into lung lavage fluid. Lavage and peripheral blood differentials were measured at 6 and 24 hours post injury and levels of key antioxidant enzymes were monitored in the lung using immunohistochemistry. We found that exposure to soot stimulated an increase in circulating neutrophils in adult rats and an increase in lavage neutrophils in neonates. LDH release was significantly increased in the lavage fluid of neonates only at the 2 hr timepoint but cellular injury was not morphologically apparent at either age. Distribution and abundance of two anti-oxidant enzymes, glutamate cysteine ligase and heme oxygenase-1 were increased in the soot exposed animals, particularly in the airway epithelium. We conclude that low PAH soot is not especially injurious to lungs of neonatal or adult rats but that exposure to ambient

levels of this type of particulate does cause changes in neutrophil abundance and increases in antioxidant enzymes. Possible additional injury in neonates is supported by increased LDH in lavage fluid. Funded by EPA RD-83241401 and NIH ES/HL 06700.

569/B521

Down-Regulation of Thrombomodulin Induces Tumorigenesis in HaCaT Cells.

Y. Kao[1], L. Wu[2,4], C. Shi[3,4], H. Sheu[5], H. Wu[3,4], G. Shi[3,4]; [1]Institute of Basic Medical Sciences, College of Medicine, National Cheng Kung University, Tainan, Taiwan, [2]Institute of Molecular Medicine, College of Medicine, National Cheng Kung University, Tainan, Taiwan, [3]Department of Biochemistry and Molecular Biology, National Cheng Kung University, Tainan, Taiwan, [4]Cardiovascular Research Center, National Cheng Kung University, Tainan, Taiwan, [5]Department of Dermatology, College of Medicine, National Cheng Kung University, Tainan, Taiwan

Thrombomodulin (TM), a type 1 transmembrane glycoprotein, is originally identified as the receptor of thrombin to generate activated protein C, which is responsible for the blockage of coagulation in endothelial cells. We previously demonstrated that TM functions as a calcium-dependent molecule to mediate cell adhesion in similar fashion to that of E-cadherin. In addition, increasing reports show that TM expression has negative correlation with tumor progression and survival rate of tumor-bearing patients. However, the role of TM in tumorigenesis remains most unknown. This study aims at investigating the role of TM in tumorigenesis. When TM expression in HaCaT cells was suppressed by short hairpin RNAs targeting to TM, E-cadherin and β-catenin proteins expression level were not altered. However, E-cadherin not only dissociated with β-catenin but also dislodged from cell-cell junction, causing the cells to have loose cell contact and assume characters of tumor cells. TM knockdown HaCaT cells had higher motility and proliferation rate than control cells. Moreover, injection of TM knockdown cells into severe combined immunodeficiency mice induced squamous cell carcinoma-like tumors which were highly proliferated, as demonstrated by proliferating cell nuclear antigen and BrdU immunostaining. Additionally, premature expression of loricrin, a marker of terminal epidermal differentiation, was observed in the lower epidermal layers in the tumor. In conclusion, we demonstrate that TM may function as a tumor suppressor via regulating cell junction, cell proliferation, and tumorigenesis.

570/B522

Metabolomic Effects of CT50 and Sub-CT50 Doses of Eastern Cottonmouth Venom on Mammalian Cells.

B. Morrill, L. Rickords; Animal, Dairy, and Veterinary Science, Utah State University, Logan, UT

The effects of snake venoms on mammalian tissues and cells have been studied for decades. However, little research has focused on the metabolic changes induced in mammalian cells by these venoms. Studying the metabolome allows us to obtain an amplified view of changes that may be occurring at the transcriptome and proteome levels because metabolome changes are a downstream effect of gene expression. The metabolome can be an important indicator of phenotype since metabolic fluxes are not only regulated by gene expression, but also by post-transcriptional and post-translational events. A cytotoxic dose (CT50) of 12.5 µg/ml of media for Eastern Cottonmouth (*Agkistrodon piscivorus piscivorus*) venom (Sigma) on Vero cells in culture was calculated using a neutral red assay. CT50 and sub-CT50 dose samples were collected for gas chromatography-mass spectroscopy (GC/MS) by treating confluent Vero cells with 12.5 µg/ml and 10.0 µg/ml of media respectively in a T75 flask for four hours prior to trypsinization and cell collection. Metabolites were then extracted from the samples and silylated prior to being analyzed by GC/MS. Results will be presented demonstrating differences in concentrations of key metabolites between both treated and control cells, and between CT50 and sub-CT50 treated cells. Further research into such metabolites could provide a deeper understanding of the cellular changes that are caused by pitviper venoms on mammalian cells.

571/B523

Analysis of Loxosceles intemedia Mello-Leitão (1934) (Araneae: Sicariidae) Male Genital Tract.

A. Margraf[1], C. Costa-Ayub[1], C. Ortolani-Machado[2], M. A. Soares[1]; [1]Biologia Estrutural, Molecular e Genética, Universidade Estadual de Ponta Grossa, Ponta Grossa, Brazil, [2]Biologia Celular, Universidade Federal do Paraná, Curitiba, Brazil

Anatomical variations of the male genital tract have been described for different species of spiders, mainly concerning to the spermatic channels. Loxosceles intermedia testes consist of a long pair of cylindrical bodies, connected to a long and coiled vas deferens by a thin duct and an enlarged ampulla. The presence of an ampulla has not yet been described in other species of spider. In the final portion of the vas deferens a secretion sheath is observed around each synspermium unit and the conjunct cell sperm more secretion is released during the copulation. Study's objective was to investigate the secretory morphology of the duct and ampulla at light (HE stained sections and PAS reaction) and scanning electron microscopy levels. Synspermia leave the testes, within a thin secretion, pass through the duct, and gain the enlarged ampulla. The duct shows a very compact structure which could be crucial to control the passage of the synspermia through this channel. Probably in the duct the synspermia pass one by one. The grooves in the epithelium of the ampulla suggest active extrusion of poorly aggregated secretion in the proximal region and a more compact secretion is seen in the distal region. The secretion of the ampulla is PAS positive and probably is essential for maintaining cell integrity. The nonsecretory epithelium of the duct could act as a selective gate for synspermia allowing a slow flux in direction to the ampulla reservatoir, as well as their interaction with the local secretion.

Leukocytes (572-579)

572/B525

Cdc42 Regulates Fcγ Receptor- mediated Phagocytosis through WASP Activation.

H. Park[1], D. Cox[1,2]; [1]Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, Yeshiva University, Bronx, NY, [2]Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, Bronx, NY

Cdc42, a Rho family GTPase, is a well-known key regulator of the actin cytoskeleton and the activation of WASP (Wiskott-Aldrich Syndrome Protein). Although several studies have demonstrated the requirement of Cdc42 in phagocytosis by dominant negative mutants, the role of Cdc42 mediated signaling pathways in phagocytosis is still unclear. Here, we demonstrate that the reduction of Cdc42 expression

using RNA-mediated interference in a macrophage cell line (RAW/LR5) results in a severely impaired ability of cells to carry out FcγR-mediated, but not CR3-mediated, phagocytosis. Phagocytic cup formation, actin assembly and the extension of protrusions in response to FcγR ligation required Cdc42 activity. A role of WASP as a downstream effector of Cdc42 during phagocytosis was explored. WASP deficient macrophages demonstrated only a partial block in phagocytosis, actin polymerization and pseudopod extension following FcγR ligation. This partial defect in phagocytosis may be due to redundancy with N-WASP that is also expressed in macrophages. Addition of wiskostatin, a WASP/N-WASP inhibitor, showed extensive inhibition of phagocytosis, actin assembly and cell extension in primary macrophages and RAW/LR5 cells identical to the phenotype seen upon reduction of Cdc42 expression. Cdc42 was required for the activation of both WASP and N-WASP during the phagocytic cup formation as determined using a conformation-sensitive antibody (CSA) against WASP/N-WASP. However, WASP was still recruited to sites beneath bound particles in the absence of Cdc42, suggesting that another signaling pathway is required for WASP recruitment. These results clearly indicate that Cdc42 is essential for activation of WASP/N-WASP during FcγR-mediated phagocytosis, which regulates actin assembly and extension of phagocytic cups by macrophages.

573/B526

RhoH Plays Critical Roles in FcεRI-dependent Signal Transduction of Mast Cells.

H. Oda[1], M. Fujimoto[2], M. S. Patrick[1,3], H. Aoki[4], M. Shirai[3], H. Suzuki[1]; [1]Department of Pathology, Research Institute, International Medical Center of Japan, Shinjuku, Japan, [2]Department of Dermatology, Kanazawa University Graduate School of Medical Science, Kanazawa, Japan, [3]Department of Microbiology, Yamaguchi University School of Medicine, Ube, Japan, [4]Cardiovascular Research Institute, Kurume University School of Medicine, Kurume, Japan

RhoH is an atypical small G protein with defective GTPase activity, that is specifically expressed in hematopoietic lineage cells. RhoH has been implicated in regulation of several physiological processes including hematopoiesis, integrin activation, and T cell differentiation and activation. In the present study, we investigated the role of RhoH in mast cells by generating RhoH knockout mice. Despite observing normal development of mast cells in vivo, passive systemic anaphylaxis and histamine release were impaired in these mice. We also observed defective degranulation and cytokine production upon FcεRI ligation in RhoH deficient bone marrow derived mast cells. Furthermore, FcεRI-dependent activation of Syk and phosphorylation of its downstream targets, including LAT, SLP76, PLCγ1 and 2 were impaired in RhoH deficient mast cells. We also found that phosphorylated RhoH associates with Syk in Fyn dependent manner. Our results indicate that RhoH regulates FcεRI signaling in mast cells by facilitating Syk activation, possibly as an adaptor molecule for Syk.

574/B527

c-Src Tyrosine Kinase Co-associates with and Phosphorylates STAT5b Which Mediates the Proliferation of Normal Human B Lymphocytes.

M. Cayer[1], M. Proulx[2,1], X. Ma[3], D. Sakac[3], J. Jean-François Giguère[2,1], M. Drouin[1], S. Néron[1,2], D. R. Branch[3,4], D. Jung[1,2]; [1]R & D, Hema-Québec, Québec, QC, Canada, [2]Biochemistry and Microbiology, Laval University, Québec, QC, Canada, [3]R & D, Canadian Blood Services, Toronto, ON, Canada, [4]Division of Cell and Molecular Biology, Toronto General Research Institute, Toronto, ON, Canada

c-Src is the normal human cellular protein homologue of the viral oncogene v-src. Recently, c-Src activity was reported to increase in CD40-activated human B lymphocytes, suggesting its involvement in proliferation. To elucidate the exact role of c-Src in this process, we investigated the effects of c-Src over-expression on normal B lymphocytes growth. B lymphocytes purified from human peripheral blood were infected with Ad5/F35 vector encoding either a constitutively active c-Src(c-Src/DP) and a dominant negative c-Src(c-Src/DN). Little variation of B lymphocytes expansion could be observed between control EYFP and c-Src/DP infected cells. In contrast, overexpression of c-Src/DN results in a 40% inhibition of B lymphocytes expansion. These results suggest that dominant negative c-Src may compete with endogenous c-Src resulting in a partial inhibition of a transcriptional pathway involved in B lymphocytes proliferation. We further demonstrate that c-Src can phosphorylate STAT5b on tyrosine 694 and that c-Src and Stat5b co-associate during B lymphocytes proliferation. These results confirm an important role for c-Src in the expansion of normal human B lymphocytes in vitro, in which c-Src may regulate STAT5b in the intracellular signalling pathway important for the proliferation of normal human B lymphocytes

575/B528

The Association between STAT5a Activation, LEF-1 Down Regulation and Leukemic Transformation in Patients with Severe Congenital Neutropenia.

K. Gupta, J. Skokowa, K. Welte; Dept of Pediatric Hematology Oncology, Hannover Medical School, Hannover, Germany

Hematological disorders are excellent models for investigating the regulation of hematopoiesis in humans. Severe Congenital Neutropenia (CN) is a heterogeneous syndrome characterized by a maturation arrest of granulopoiesis at the level of the promyelocytes with no mature neutrophils in the peripheral blood. Even though G-CSF treatment results in increased neutrophil numbers in more than 90 % of cases, CN patients are found to be at high risk of developing AML (~ 20 %). Since AML is not observed in cyclic neutropenia patients treated with G-CSF, an underlying defect down stream of G-CSF signaling rather than G-CSF therapy per se predisposes to malignant transformation in CN. STAT5 is activated by G-CSF signaling pathway and has also been found to be activated in AML. Recently we found that downregulation of LEF-1 transcription factor and its target gene C/EBPα are a common pathomechanism for CN. Therefore, we investigated the effects of G-CSF on the phosphorylation status of STAT5a in CN and whether it leads to the down modulation of LEF1 expression and functions. We found that G-CSF stimulation resulted in a significantly higher phosphorylation of STAT5a in CD34+ cells of CN patients as compared to healthy volunteers. Transduction of constitutive active STAT5a (STAT5a 1*6) in CD34+ cells of healthy individuals resulted in significant downregulation of LEF1 expression in a dose dependent manner. A screen of 10 kb upstream region of LEF1 gene revealed two putative STAT5 binding sites, confirmed by ChIP assay using the nuclear extracts of CD34+ cells. We found enhanced and prolonged STAT5a binding to the LEF-1 promoter in G-CSF treated CD34+ cells from CN patients, as compared to healthy individuals. Additionally, transfection of CD34+ cells with LEF-1 cDNA resulted in elevation of LEF-1 promoter activity and co-transfection with STAT5a 1*6 significantly disrupted LEF-1-dependent activation of LEF-1 promoter. Moreover STAT5a 1*6 severely abrogated the LEF1 dependent

regulation of C/EBPα gene promoter. Taken together phosphorylation of STAT5 is upregulated in hematopoietic progenitors of CN patients and downstream effects of activated STAT5a may contribute to the malignant transformation of myelopoiesis in CN.

576/B529

MCP-1 Enhances Monocyte Adhesion to VCAM-1 without Inducing a Change in VLA-4 Affinity.

C. Chu, F. Rico, V. T. Moy; Physiology and Biophysics, Unversity of Miami: Miller School of Medicine, Miami, FL

Monocyte Chemoattractant Protein-1 (MCP-1) is a chemokine which induces monocyte migration across the arterial wall into the intima, leading to the accumulation of monocytes which will eventually form atherosclerotic plaques. MCP-1 induces monocyte migration by enhancing the adhesion of integrins expressed on the leukocyte cell surface to ligands expressed on the vascular endothelium. Previous work suggests that MCP-1 enhances the adhesion of the integrins Very Late Antigen-4 (VLA-4) and Lymphocyte Function-associated Antigen 1 (LFA-1) to their respective ligands: Vascular Cell Adhesion Molecule-1 (VCAM-1) and Intercellular Cell Adhesion Molecule-1 (ICAM-1), respectively. It is unclear whether MCP-1 affects both VLA-4 and LFA-1, or only one of the integrins. Furthermore, there is some debate whether chemokines enhance leukocyte adhesion through integrin affinity or avidity regulation. In this study, the Atomic Force Microscope (AFM) was used to characterize the effect of MCP-1 on the adhesive properties of VLA-4 and LFA-1 in THP-1 cells, a human monocytic cell line. THP-1 cells were immobilized to the force transducer and then lowered onto the functionalized substrate for 0.1s of contact time; and the work of detachment, maximum detachment force, and maximum detachment distance were determined. MCP-1 significantly enhanced THP-1 adhesion to VCAM-1 increasing the work of detachment, maximum detachment force, and also the maximum detachment distance. In addition, single molecule force measurements were also acquired to examine whether MCP-1 enhances VLA-4 affinity to VCAM-1. In conclusion, when THP-1 cells were only stimulated with MCP-1, VLA-4 adhesion to VCAM-1 was enhanced by avidity regulation, but had no apparent effect on LFA-1 adhesion at short contact times.

577/B530

N-acetylcysteine Promotes Apoptotic Cell Clearance Resulting in Suppressing Proinflammatory Mediators and Enhancing TGF-β in Intense Lung Inflammation.

C. Moon, H. Park, J. R. Han, J. L. Kang; Physiology, Ewha Womans University, School of Medicine, Seoul, South Korea

Objective: to determine the in vivo effects of an antioxidant N-acetylcysteine (NAC), on apoptotic cell clearance by alveolar macrophages and inflammatory mediator productions in intense lung inflammation associated with oxidative stress. Methods: Neutrophil accumulation, oxidant production and apoptosis in bronchoalveolar lavage (BAL) cells were examined after i.t. LPS treatment. NAC was i.p. administered to study the effects on apoptotic cell clearance by alveolar macrophages and inflammatory mediator productions. Results: Neutrophil accumulation and ROS activity peaked at 3 d post-LPS. LPS-treated mice showed clear apoptosis of BAL cells and enhanced in vivo and in vitro apoptotic cell clearance by alveolar macrophages. NAC further enhanced in vivo and in vitro apoptotic cell clearance by alveolar macrophages and inhibited apoptosis of BAL cells. NAC inhibited proinflammatory mediator secretion but further enhanced an anti-inflammatory mediator, TGF-β1, in BAL fluid or in alveolar macrophage culture. In addition, the TNF-α secretion in alveolar macrophages from LPS-treated mice with NAC or without NAC was further reduced when exposed to in vitro apoptotic cells, but the TGF-β1 secretion was further enhanced. The Rho activation in lung tissue from LPS-treated mice was suppressed by NAC. The phosphorylation and degradation of IκB-α were not affected at 3 d post-LPS. Conclusion: NAC further enhances apoptotic cell clearance and concomitantly reduces TNF-α but increases TGF-β1 secretion in LPS-induced intense lung inflammation.

578/B531

Regulation of Osteoclast Differentiation by RANKL-induced Oxidation of SHP-1.

H. Shon[1], Y. Kim[2], Y. Noh[1], J. Baek[1], J. Jang[1], J. Min[1], Y. Yang[2], T. Chang[1,2,3]; [1]Division of Life and Pharmaceutical Science, Ewha Womans University, Seoul, South Korea, [2]Institute of Molecular Life Science and Technology, Ewha Womans University, Seoul, South Korea, [3]College of Pharmacy, Ewha Womans University, Seoul, South Korea

Osteoclasts play a critical role in bone remodeling by resorbing the bone matrix. Receptor activator of NF-κB ligand (RANKL) is necessary for osteoclast differentiation from monocyte/macrophage lineage cells. RANKL-induced generation of intracellular reactive oxygen species (ROS) modulates signaling cascades responsible for osteoclast differentiation, but how ROS affect signaling pathways is largely unknown. Here, we show that RANKL-stimulated production of ROS cause inhibition of SH2 domain-containing protein tyrosine phosphatases-1 (SHP-1) by oxidation of its catalytic cysteine residue. Treatment of cells with an inhibitor of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase prevents ROS production, SHP-1 oxidation, and osteoclastogenesis. Furthermore, RANKL-induced tyrosine phosphorylation of spleen tyrosine kinase (Syk) and bruton's tyrosine kinase (Btk) is regulated by a redox-dependent manner. Taken together, our results suggest that RANKL-mediated ROS production leads to oxidation of SHP-1 that is followed by the promotion of osteoclast differentiation.

579/B532

Lectin Isolated from Momordica Charantia Seed Activates Murine B Cell.

T. Haga[1], L. Huang[1], T. Adachi[1], Y. Shimizu[1], Y. Goto[1], J. Toyama[2], H. Tanaka[3], R. Akashi[3], A. Sawaguchi[4], H. Iwata[5]; [1]Veterinary Microbiology, University of Miyazaki, Miyazaki, Japan, [2]Japan Science and Technology Agency Innovation Satellite Miyazaki, Miyazaki, Japan, [3]Frontier Science Research Center, University of Miyazaki, Miyazaki, Japan, [4]Anatomy, Ultrastructural Cell biology, Faculty of Medicine, University of Miyazaki, Miyazaki, Japan, [5]Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan

Momordica charantia lectin (MCL) is a galactose-specific protein found in the seeds of bitter gourd that has been the subject of active research for two decades. MCL is a tetrameric glycoprotein with α2β2 type subunit architecture that exhibits agglutinating activity toward human type-O red blood cells. We previously demonstrated the immunoadjuvant activity of MCL in a mouse model. To further characterize the immunostimulatory effect of MCL, we studied its effect on murine lymphocytes in vitro. CD19-positive B cells and CD3e-positive T cells were isolated from spleen of normal BALB/c mice using B cell and Pan T cell isolation kit, respectively. Cell proliferation was

measured by ELISA with the incorporation of BrdU. Flow cytometry analysis was performed to assess MCL binding specificity to splenocytes using FITC-labeled MCL and labeled antibodies against lymphocyte surface markers. Immunogloblin (Ig) production in the supernatant of cell cultures treated with MCL was measured by ELISA. Cell proliferation assay showed that MCL specifically stimulates B cells and that this activation occurred without T cell help. Flow cytometry analysis revealed that FITC-labeled MCL selectively binds to CD19-positive B cells, which were suppressed by specific sugars, including galactose. Moreover, MCL triggered splenocytes in culture to produce dominant non-specific IgM, as detected in the supernatant. In conclusion, this is the first study to demonstrate that MCL is a T cell-independent B cell activator and a polyclonal Ig inducer. As this study challenges the classic view of the majority of lectins as T cell mitogens, these results may also have specific implications for the research of B cell development.

Neurotransmitters, Peptides, and Receptors (580-588)

580/B541

Functional Expression of β2 Nicotinic Acetylcholine Receptor Construct with α-Bungarotoxin Sensitive Neuronal Subunits in Xenopus Oocytes.

E. C. Gutierrez[1], G. Chew[2], E. Hawrot[2]; [1]Industrial Biotechnology, University of Puerto Rico - Mayaguez, Mayaguez, PR, [2]Molecular Pharmacology, Physiology and Biotechnology, Brown University, Providence, RI

Neuronal nicotinic acetylcholine receptors are ligand gated ion channels found in the central nervous system. There are 9 α and 3 β subunits that can form a pentameric receptor. Acetylcholine (ACh), a nAChR agonist, can bind to the receptor between the α and β subunit. Neuronal nAChRs, unlike muscle type nAChRs, are not blocked by the neurotoxin α-Bungarotoxin (Bgtx). Previously, using a mutagenic screen, our laboratory isolated an α3 subunit that conferred Bgtx sensitivity to the neuronal receptor when functionally expressed with β2 and β4 subunits. We called this chimeric subunit α3/α1[5]. Co-expression of the α3/α1[5] chimera together with β4 subunit has been replicated, however expression with wild type β2 has not been successful. It was hypothesized that the original β2 used in the initial study might have had an unknown single point mutation that led to the functional expression. To address this, we sequenced this original β2 and compared it to the wild type β2 used in the subsequent replications. Sequencing results showed that the original β2 is identical to wild type β2. Despite the similarities, we proceeded to attempt functional expression of the α3/α1[5] chimera with the original β2. We extracted and purified the β2 DNA, transcribed it into cRNA, and co-injected the α and β subunits into Xenopus laevis oocytes. Two-electrode voltage clamp electrophysiology was used to record ACh-evoked responses from the oocytes. Electrophysiologic results showed no response to ACh which strongly suggests that there is no functional expression of the β2 subunit with the α3/α1[5] mutant in Xenopus oocytes. We are now studying the possibility of a β2 and β4 chimera in order to better understand their individual roles in the functional expression of neuronal nicotinic aceytlcholine receptors.

581/B542

Fluorophore-assisted Light Inactivation of Recombinant 5-HT3A Receptors.

R. A. Morton[1,2], G. Luo[1], M. Davis[1], T. G. Hales[2], D. M. Lovinger[1]; [1]NIAAA, National Institutes of Health, Rockville, MD, [2]Physiology Pharmacology, George Washington University, Washington, DC

In recent years the development of fluorescent molecules has made it possible to visualize individual proteins and study such processes such as protein trafficking, localization, and function. The aim of this study was to use a technique known as fluorophore-assisted light inactivation (FALI) to specifically inhibit the ionotropic 5-HT3A receptor function in a spatially and temporally restricted manner. To accomplish this, the 5-HT3A receptor was tagged with the α-bungarotoxin binding sequence (BBS) on the extracellular c-terminus of the protein, thus enabling only cell surface receptors to be labeled with fluorescently conjugated α-bungarotoxin. Labeling of BBS containing 5-HT3A receptors with conjugated α-bungarotoxin did not significantly alter receptor distribution or function in HEK 293 cells or a neuroblastoma cell line N1E-115 that natively express 5-HT3A receptors. Recombinant 5-HT3A receptors expressed in HEK or N1E cells exhibited constitutive internalization. Following α-bungarotoxin labeling and exposure to light at wavelengths that excite the conjugated fluorophore the constitutive internalization of 5-HT3A receptors was inhibited. Furthermore, excitation of the fluorophore substantially reduced 5-HT evoked peak current amplitude. Receptor inactivation was reduced by exposure to an oxygen radical scavenger sodium azide during FALI, suggesting the involvement of oxygen radicals. These data demonstrate a novel way to inhibit function and internalization of 5-HT3A receptors in a spatially restricted manner by only labeling the receptors on the cell surface, and also in a temporally restricted manner depending on when the fluorophore is exposed to excitatory light.

582/B543

A Nucleostemin Family GTPase, NS3, Acts in Serotonergic Neurons to Regulate Insulin-producing Neurons and Control Body Size.

D. D. Kaplan[1,2], G. Zimmermann[1], K. Suyama[1], D. Y. Gui[1], E. E. Lee[1], S. Sood[1], T. Meyer[2], M. P. Scott[1]; [1]Developmental Biology, Genetics, and Bioengineering, HHMI/Stanford University School of Medicine, Stanford, CA, [2]Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA

Growth and body size are regulated by the central nervous system (CNS), integrating the genetic developmental program with assessments of an animal's current energy state and environmental conditions. CNS decisions are transmitted to all cells of the animal by insulin/insulin-like signals. The molecular biology of the CNS growth control system has remained for the most part elusive. Here we identify NS3, a Drosophila nucleostemin family GTPase, as a powerful regulator of body size. ns3 mutants reach <60% of normal size and have fewer and smaller cells, but exhibit normal body proportions. NS3 does not act cell autonomously, but instead acts at a distance to control growth. Rescue experiments were performed by expressing wild-type ns3 in many different cells of ns3 mutants. Restoring NS3 to only 106 serotonergic neurons rescued global growth defects. These neurons are closely apposed with those of insulin-producing neurons, suggesting possible communication between the two neuronal systems. In the brains of ns3 mutants, excess serotonin and insulin accumulate, while peripheral insulin pathway activation is low. Peripheral insulin pathway activation rescues the growth defects of ns3 mutants. The findings suggest that

NS3 acts in serotonergic neurons to regulate insulin signaling and thus exert global growth control. Using a combination of in vivo RNAi screening and proteomic approaches, we have identified other key molecules acting at the interface between serotonergic and insulin-producing neurons. We are working to determine the mechanism through which these components coordinate cell growth and body size.

583/B544

Nitric Oxide and Muscarinic Receptor-mediated Signaling: Influence on Calcium Oscillations.

H. Wang, B. A. Wheelden, A. C. Growcock, A. L. Martin, Y. Huang, R. S. Aronstam; Biological Sciences, Missouri University of Science & Technology, Rolla, MO

The effects of nitric oxide on calcium oscillations elicited by low concentrations of a muscarinic agonist were examined in CHO cells stably transfected with the gene for the human M3 receptor. NO targets include several synaptic receptor-mediated signaling processes. Agonist stimulation of M3 muscarinic acetylcholine receptors leads to activation of phospholipase Cβ mediated by Gq,11 proteins, leading to the production of the second messengers inositol triphosphate (IP3) and diacylglycerol. IP3 acts on IP3 receptors in the endoplasmic reticulum to release Ca2+ into the cytosol; this depletion of calcium stores stimulates calcium influx. At near threshold agonist concentrations, a cyclical release and re-uptake of calcium from intracellular stores (calcium oscillations) is the predominant response. METHODS: Calcium influx was eliminated by the use of calcium-free medium; fura-2 fluorescence was used to quantify cytosolic calcium concentration; carbamylcholine was used to activate muscarinic receptors; nitric oxide was provided by application of 4.6 or 8.3 mM freshly-prepared S-nitrosylglutathione (SNG) for 10 min prior to measuring muscarinic responses. Calcium release from intracellular stores was characterized by carbamylcholine sensitivity, maximum initial response, and the presence, amplitude and decay of calcium oscillations. RESULTS: Pretreatment with nitric oxide donor decreased the amplitude of the initial Ca2+ peak by ≈15%, but increased the proportion of cells displaying oscillatory behavior from <10% to >90%. Changes were not observed in oscillation frequency or oscillation decay. The threshold for the elicitation of Ca2+ responses by carbamylcholine increased, consistent with a NO-mediated subsensitivity. The average delay in the manifestation of the Ca2+ response increased markedly from 5.3 sec to 8.0 and 12.2 sec in the presence of 4.3 and 8.5 mM SNG, respectively. CONCLUSIONS: Nitric oxide modulates the ability of M3 muscarinic receptors to activate calcium signaling pathways as indicated by an enhanced development of calcium oscillations. These changes may reflect changes in the potency of the muscarinic ligand. Supported by the MST cDNA Resource Center (www.cdna.org).

584/B545

Molecular Cloning and Functional Expression of a Novel Human CGRP Receptor.

J. P. Vu[1,2], Y. Lu[1,2], S. Wank[3], J. Pisegna[1,2]; [1]Medicine, UCLA, Los Angeles, CA, [2]Gastroenterology, Veterans Administration GLHS, Los Angeles, CA, [3]Digestive Diseases, National Institutes of Health, Bethesda, MD

Calcitonin Gene Related Peptide (CGRP) is a 37 amino acid neuropeptide that belongs to the calcitonin superfamily, inducing extensive physiological effects in the brain, vascular system, and gastrointestinal tract. The receptors for CGRP have been localized by immunohistochemistry in sensory nerves and CNS neurons and highly expressed throughout the brain and gastrointestinal tract in rats. Previously a high affinity receptor for CGRP included the Calcitonin Receptor-Like Receptor (CRLR) requires co-expression with RAMP proteins for expression. We have previously reported the molecular characterization of a novel receptor in the Class-I-superfamily of G-Protein Coupled-Receptors(GPCRs) with high affinity to both CGRP and adrenomedulin in rats. Based on this, our current study is to clone the human homologue of this high affinity receptor and to explore its pharmacological characterization as a candidate CGRP2 receptor. The receptor was cloned from a human genomic DNA library using degenerate primers flanking the 5' and 3' coding sequence of the rat CGRP receptor. The cloned human receptor was subcloned in pCDL-Sra/Neo and stably transfected into NIH 3T3 cells. The receptor was sequenced and pharmacologically characterized by evaluating the cAMP stimulation in response to CGRP, adrenomedulin with and without the partial CGRP-receptor antagonist CGRP8-37. The cloned human receptor responded to the stimulation of CGRP, adrenomedulin, and amylin in a dose-dependent fashion. The effective concentration 50% of maximum(EC50) response was highest with adrenomedulin at a concentration of 10-8M, following with CGRP at 10-6M. The effective concentration of CGRP8-37 to show antagonizing function was 10-5M with CGRP and 10-7M with adrenomedulin. Based on the pharmacological characterization of this receptor showing greater efficacy and potency for adrenomedullin>CGRP and that there was partial antagonism to the CGRP receptor partial antagonist CGRP8-37 we propose that this receptor belongs to a novel Class-IG Protein-Coupled-Receptor superfamily. These results will permit a more detailed determination as to the localiazation and physiological function of this novel receptor.

585/B546

Structural Study Reveals That the High-Affinity Ephrin-binding Channel of the EphA4 Receptor Can Be Targeted by Two Small Molecule Antagonists.

J. Shi[1], H. Qin[1], E. B. Pasquale[3,4], R. Noberini[3,5], J. Song[1,2]; [1]Department of Biological Sciences, National University of Singapore, Singapore, Singapore, [2]Department of Biochemistry, National University of Singapore, Singapore, Singapore, [3]Burnham Institute for Medical Research, La Jolla, CA, [4]Pathology Department, University of California San Diego, La Jolla, CA, [5]Department of Biochemistry and Molecular Biology, University of Parma, Parma, Italy

The Eph receptor tyrosine kinases regulate a variety of physiological and pathological processes not only during development but also in adult organs, and therefore represent a promising class of drug targets. The EphA4 receptor plays important roles in the inhibition of the regeneration of injured axons, synaptic plasticity, platelet aggregation and likely in certain types of cancer. Here we report the first crystal structure of the EphA4 ligand-binding domain, which adopts the same jellyroll β-sandwich architecture as previously shown for EphB2 and EphB4. The similarity with EphB receptors is high in the core β-stranded regions, whereas large variations exist in the loops, particularly the D-E and J-K loops, which form the high-affinity ephrin binding channel. We also used isothermal titration calorimetry, NMR spectroscopy and computational docking to characterize the binding to EphA4 of two small molecules, 4- and 5-(2,5 dimethyl-pyrrol-1-yl)-2-hydroxy-benzoic acid which antagonize ephrin-induced effects in EphA4-expressing cells. We show that the two molecules bind to the EphA4 ligand-binding domain with Kd values of 20.4 and 26.4 μM, respectively. NMR HSQC titrations revealed that upon binding, both molecules

significantly perturb EphA4 residues Ile31-Met32 in the D-E loop, Gln43 in the E β-strand and Ile131-Gly132 in the J-K loop. Molecular docking shows that they can occupy a cavity in the high-affinity ephrin binding channel of EphA4 in a similar manner, by interacting mainly with the EphA4 residues in the E strand, D-E and J-K loops. However, many of the interactions observed in Eph receptor-ephrin complexes are absent, which is consistent with the small size of the two molecules and may account for their relatively weak binding affinity. Thus, our studies provide the first published structure of the ligand binding domain of an EphA receptor of the A subclass. Furthermore, the results demonstrate that the high-affinity ephrin-binding channel of the Eph receptors is amenable to targeting with small molecule antagonists and suggest avenues for further optimization.

586/B547

The pERK Expression in the Rat Brainstem Induced by Capsaicin and Formalin Stimulation of Rat Conical Papillae.

S. Kumabe[1], M. Nakatsuka[1], S. Jue[2], K. Takama[1], J. Shin[2], Y. Iwai[1]; [1]Osaka Dental University, Hirakata, Japan, [2]Kyung-Hee University, Seoul, South Korea

Objective: ERK (extracellular signal-regulated kinase) is a member of the MAPK (mitogen-activated protein kinase) family. The MAPK signaling pathway is usually activated by nociceptive stimuli. We investigated phospho-ERK (pERK) expression in the rat brainstem following stimulation of rat conical papillae by capsaicin or for-malin. Materials and Methods: 12 adult male rats (Sprague-Dawley; Orient Bio) were anesthetized with sodium pentobarbital (Nembutal; 70mg/kg, i.p.; Dainippon Sumi-tomo Pharma). Capsaicin (Sigma)-0% (normal saline, 10µl; control 1) and -0.1% (capsaicin diluted with 70% ethanol, 10µl; experimental 1) solutions or formalin-0% (normal sa-line, 10µl; control 2) and -5%(10µl; experimental 2) solutions were soaked with filter paper sheets, and applied onto rat conical papillae for 5 minutes. The brainstems were dissected out from S-D rats, frozen-sectioned, and immunohisto-chemically stained with anti-pERK Ab (first Ab) and anti-rabbit IgG Ab (second Ab) by the conventional ABC methods. The pERK-immunoreactive cells in counter-stained specimens were analyzed. Results: Following capsaicin stimulation, pERK-immunoreactive cells were observed in the trigeminal nucleus caudalis (significant, $p<0.05$), area postrema, nucleus of the solitary tract (significant, $p<0.05$) and reticular nucleus of the experimental 1 group; very few were observed in the control 1 group. Following formalin stimulation, the pERK-positive cells were recognized in the spinal trigeminal nucleus caudal part (significant, $p<0.05$) and medullary reticular nucleus(significant, $p<0.01$). Conclusion: The MAPK activates nociceptive neurons and elicits pain hypersensitivity by capsaicin and formalin application onto rat conical papillae, but the stimuli induce differ MAPK activationin the rat brainstem.

587/B548

Signaling of High Affinity Choline Transport.

J. Xie, Q. Guo; Department of Physiology, The University of Oklahoma Health Sciences Center, Oklahoma City, OK

The high-affinity choline transporter-1 (CHT1) is a Na+ and Cl--dependent, hemicholinium-3 (HC-3) sensitive choline transporter. Unlike other neurotransmitter transporters, CHT1 is predominantly localized inside the cell, suggesting that internalization/endocytosis and intracellular trafficking of CHT1 may play a crucial role in regulating choline uptake. However, the molecular determinants that control the internalization/endocytosis, subcellular distribution, and intracellular trafficking dynamics of CHT1 remain undefined. We have recently found that Par-4 (prostate apoptosis response-4), a leucine-zipper protein associated with cholinergic signaling and neurodegeneration, interacts directly with CHT1, and this interaction seems to play a critical role in determining the cell surface levels of CHT1. Mapping studies indicated that the carboxyl terminal half of Par-4 was physically involved in interacting with CHT1. Recently, we also found that a peptide from C-terminal cytosolic tail of CHT1 (514FDAVVARHSEENMDKTILVKNENIKC538) that was covalently linked to Sulfolink Coupling Gel was able to bind to Par-4 in pull down assays. Death-associated protein kinase (DAPK) is a multidomain calcium/calmodulin-dependent serine/threonine protein kinase whose native substrates are not well defined. We found that Par-4 was phosphorylated by DAPK in a calcium-dependent manner in vitro and in vivo. In phosphorylation assays, we found that S249A mutation of Par-4 effectively eliminated 32P incorporation, indicating that serine 249 of Par-4 is a specific phosphorylation site for DAPK. Of importance, S249A mutation of Par-4 not only blocked phosphorylation of Par-4 by DAPK, but also significantly enhanced Par-4/CHT1 interaction, indicating that phosphorylation of Par-4 by DAPK may function to impair direct interaction between Par-4 and CHT1. Thus, DAPK mediated phosphorylation of Par-4 may represent a novel mechanism coupled to regulated expression of CHT1 and high affinity choline uptake at neuronal surface.

588/B549

Localization of NOMPC Channel in the Mechanosensory Cilia of Drosophila.

Y. Cha, S. Moon, Y. Chung; University of Seoul, Seoul, South Korea

The Drosophila TRPN ion channel subunit, NOMPC, is required for the mechanoreceptor current in tactile bristles. In auditory chordotonal (ch) neurons of the fly's antennal Johnston's organ (JO), NOMPC as well as TRPV are required for mechanotransduction. Biophysical evidence suggested that TRPN may be required for the primary transduction channel, whereas TRPV for generation of the action potentials. However the subcellular location of NOMPC, key for understanding its exact role in transduction, has never been established. Here, we generated anti-NOMPC antibody, and localized NOMPC in mechanosensory cilia. NOMPC is highly enriched in the distal tip of sensory cilia, as predicted for a mechanotransducer channel. In ch neurons, the TRPN and TRPV channels are segregated respectively into distal and proximal ciliary zones, supporting a model of auditory chordotonal transduction in which the TRPN and TRPV channels play sequential roles in generating and amplifying the receptor potential. NOMPC localization in various mechanosensory-defective mutants will also be presented.

Signal Transduction in Development (589-613)

589/B551

The Arf Gap Centaurin Alpha-1 Regulates Arf6, Rac1 and Erk in Developing Neurons.

D. Hill, J. Larimore, C. Moore, S. Worth, C. Chapleau, L. Pozzo-Miller, A. Theibert; University of Alabama at Birmingham, Birmingham, AL

Centaurin alpha-1, recently renamed ADAP1, is a candidate neuronal PI 3-kinase target that localizes to dendrites, dendritic spines and synapses, and is required for neuronal differentiation and spine morphogenesis in cultured neurons, with the effects of ADAP1 shown to require its Arf GAP activity. Overexpression studies in COS cells suggest that ADAP1 acts as a GAP for Arf6. In the current study we show that ADAP1 colocalizes extensively with Arf6, and expression of ADAP1 reduces Arf6 GTP levels, while siRNA knock down increases Arf6 GTP levels in primary cultured hippocampal neurons. Next we show that the constitutively GTP bound mutant Arf6Q67L, which reduces dendritic branching on its own, is able to reverse the effects of ADAP1 enhancement of dendritic branching. Conversely, expression of the GDP-bound mutant, Arf6T27N, which enhances branching and outgrowth on its own, can reverse the loss of dendrite outgrowth and branching induced by expression of the GAP inactive ADAP1 mutant or siRNA mediated knockdown of ADAP1. Previous studies have also shown that Rac1 is downstream of Arf6. Here we show that Rac1 activation is enhanced in ADAP1 over-expressing neurons and this activation depends on ADAP1 GAP activity. Rac1 activation is reduced in neurons in which ADAP1 levels are knocked down by siRNA. These data suggest that ADAP1 functions through GAP-dependent regulation of Arf6, and possibly through activation of Rac1, to modulate dendritic differentiation. Studies are underway to determine the localization of endogenous Arf6 in dendritic spines and the role of Arf6 in ADAP1 regulation of spine morphogenesis as well.

590/B552

SH2B1β Enhances Fibroblast Growth Factor 1 (FGF1)-induced Neurite Outgrowth through Promoting FGF1 Signaling and Increasing FGF Receptor 1 Expression.

L. Chen, W. Lin, C. Chen; Institute of Molecular Medicine, National Tsing Hua University, Hsinchu, Taiwan

Neuronal differentiation is a tightly regulated process during embryonic development. However, the mechanism of differentiation is not completely understood. PC12 cell is a well-defined model for studying the effect of neurotrophin during neuronal differentiation. We have found that SH2B1β, a signaling adaptor protein, enhances Fibroblast Growth Factor 1 (FGF1)-induced neurite outgrowth in PC12 cells. We set out to determine whether SH2B1β would regulate FGF1 signaling. Our data showed that FGF1-induced phosphorylation level of ERK1/2 and AKT is enhanced and prolonged in cells stably expressing GFP-SH2B1β(PC12-SH2B1β) compared with the control cells (PC12-GFP). Results from inhibitor assays showed that when MEK/ERK is inhibited, FGF1-induced neurite outgrowth is completely blocked. In contrast, inhibiting AKT activity does not block FGF1-induced neurite outgrowth. These data suggest that SH2B1β regulates FGF1-induced neurite outgrowth in PC12 cells mainly through MEK-ERK1/2 pathway. STAT3 has been identified as a novel transcription factor downstream of MEK-ERK1/2 signaling. In this study, we have shown that Ser-727 phosphorylation of STAT3 is enhanced and prolonged in PC12-SH2B1β compared with PC12-GFP cells. We also provide evidence suggesting that FGF1-induced pSer-727 STAT3 is mediated though MEK/ERK but not PI3K/AKT pathway. To determine the downstream target of STAT3 that is affected by SH2B1β, we found that the FGF1-induced expression of an immediately early gene downstream of MEK-ERK1/2, Egr-1, is significantly higher in PC12-SH2B1β than that in PC12-GFP. Interestingly, the downstream gene of Egr-1, p35, is not affected. These results imply that SH2B1β regulates FGF1/ERK pathway to activate transcription factors, STAT3 and Egr-1, and the downstream genes expression required for neurite outgrowth in PC12 cells. In addition, nerve growth factor (NGF)- and FGF1-induced phosphorylation of ERK1/2, AKT and Ser-727 STAT3 was compared. The results showed differential regulation of SH2B1β in FGF1 versus NGF signaling and gene expression. Taken together, our data suggest that the SH2B1β regulates FGF1 and NGF-induced neurite outgrowth through different mechanisms.

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A dsRNA Genetic Screen to Identify Regulators of Intracellular Traffic in the Notch Signalling Pathway.

S. Le Bras, G. Kriegel, F. Vignaux, R. Le Borgne; IGDR-CNRS UMR 6061-Faculté de Médecine, Rennes, France

The Notch signalling pathway is involved in numerous cellular processes during development and throughout adult life. Although ligands and receptors are largely expressed in the whole organism, the activation of Notch receptors only takes place in a subset of cells and/or tissues and is accurately regulated in time and space. Previous studies have demonstrated that endocytosis and recycling of both ligands and/or receptors is essential for this regulation. This is particularly well illustrated in the Drosophila sensory organ lineage in which a single precursor cell, called pI, undergoes a stereotyped series of four asymmetric cell divisions to generate five different cells, four cells composing a mechanosensory bristle and a glial cell. Indeed, endocytosis followed by endosomal sorting of both the Notch receptor and its ligands is an essential mechanism by which Notch-mediated signalling regulates cell fate acquisition following asymetric cell divisions. Nonetheless very little is known about the regulators and membrane compartments involved in these processes. In order to identify new regulators of receptor and ligand intracellular traffic, we have undertaken a tissue-specific dsRNA genetic screen against candidates potentially involved in endocytosis and recycling within the endolysosomal pathway. Taking advantage of the yeast UAS/GAL4 system adapted to Drosophila transgenic lines, dsRNA against 354 candidates was induced in the Drosophila sensory organ lineage. As Notch signalling regulates binary cell fate acquisition during sensory organ development, Notch gain- or loss-of-function phenotypes can be easily observed in adult sensory organs. More than 80 genes have been identified from our primary screen and we will present the strategies undertaken to validate their role in the intracellular traffic of Notch signalling pathway components.

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Neuralized Contains a Phosphoinositide-binding Motif Required Downstream of Ubiquitination for Delta Endocytosis and Notch Signaling.

L. Skwarek[1,2], M. Garroni[1,2], C. Commisso[1,2], G. L. Boulianne[1,2]; [1]Dev & Stem Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada, [2]Molecular Genetics, University of Toronto, Toronto, ON, Canada

The Notch pathway is a widely studied intercellular signaling pathway responsible for the determination of cell fate, cell differentiation and boundary formation. Several studies have shown that the pathway can be regulated by endocytosis of both the ligand and receptor. Endocytosis of the Drosophila ligands, Delta and Serrate, is required in the signaling cell for signal initiation and requires one of two ubiquitin ligases, Neuralized or Mind bomb. We have focused on understanding the role of Neuralized in ligand internalization and Notch signaling. Neuralized encodes a highly conserved protein that consists of two Neuralized Homology Repeats (NHR1 and NHR2) and a carboxy terminal RING finger domain. Both the NHR domains and the RING finger are essential for function in vivo: the RING finger confers E3 ubiquitin ligase activity and NHR1 is required to bind Delta. Recently, we have also identified an interaction between Neuralized and phosphoinositides, modified membrane lipids that mediate membrane trafficking and signalling. We find that interactions between phosphoinositides and Neuralized contribute to the membrane localization of Neuralized in the absence of Delta, and that the phosphoinositide-binding motif is required to endocytose Delta downstream of Delta ubiquitination. We also find that this interaction may also be important for vertebrate Neuralized function. Taken together, our results demonstrate that through interactions with Neuralized, phosphoinositides regulate Delta endocytosis and by extension, Notch signal transduction. Current efforts are aimed at understanding whether the interaction between Neuralized and phosophoinositides is required for other aspects of Delta trafficking.

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Critical Role of Phosphatidylinositol 4-phosphate 5-kinase 1α in Extracellular Calcium-induced Keratinocyte Differentiation.

Z. Xie[1], S. Chang[1], S. Pennypacker[1], E. Liao[2], D. Bikle[1]; [1]Endocrine Unit, Veterans Affairs Medical Center, Northern California Institute for Research and Education and University of California, San Francisco, San Francisco, CA, [2]Institute of Metabolism and Endocrinology, The Second Xiang-Ya Hospital, Central South University, Changsha, China

Extracellular calcium (Cao) is a major regulator of keratinocyte differentiation, but the mechanism is unclear. We have previously demonstrated that Cao-induced keratinocyte differentiation requires an intracellular calcium rise caused by phosphatidylinositol 3-kinase (PI3K) dependent activation of phospholipase C-γ1 (PLC-γ1) via the production of phosphatidylinositol 3,4,5-bisphosphate [PI(3,4,5)P_3]. Since both PI3K and PLC-γ1 in the plasma membrane utilize phosphatidylinositol 4,5-bisphosphate [PI(4,5)P_2] as their substrate, stimulation of PI(4,5)P_2 synthesis in the plasma membrane would be a critical step to sustain the activities of PI3K and PLC-γ1. In this study we sought to determine the role of phosphatidylinositol 4-phosphate 5-kinase 1α (PIP5K1α), a major enzyme synthesizing PI(4,5)P_2 in the plasma membrane, in mediating the ability of Cao to induce keratinocyte differentiation. We found that treatment of human keratinocytes in culture with calcium resulted in increased PIP5K1α level and activity, as well as PI(4,5)P_2 level, binding of PI(3,4,5)P_3 to and activation of PLC-γ1 with the resultant increase in inositol 1,4,5-trisphosphate (IP_3) and intracellular calcium (Cai). Knockdown of PIP5K1α in human keratinocytes blocked Cao-induced increases in the binding of PI(3,4,5)P_3 to PLC-γ1, PLC-γ1 activity, levels of PI(4,5)P_2, IP_3 and Cai, and induction of keratinocyte differentiation markers. Co-immunoprecipitation and confocal studies revealed that Cao stimulated PIP5K1α recruitment to the E-cadherin/catenin/PI3K complex in the plasma membrane. These results indicate that following calcium stimulation, PIP5K1α is recruited by the E-cadherin-catenin complex to the plasma membrane where it provides the substrate PI(4,5)P_2 for both PI3K and PLC-γ1 critical for the generation of the second messengers IP_3 and Cai required for Cao-induced keratinocyte differentiation.

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Wnt3a-mediated Formation of Phosphatidylinositol 4,5-bisphosphate Regulates LRP6 Phosphorylation.

W. Pan, D. Wu; Yale University, New Haven, CT

The canonical Wnt-β-catenin signaling pathway is initiated by induction of phosphorylation of one of the Wnt receptors, low density lipoprotein receptor-related protein (LRP) 5/6, at Thr1479 and Ser1490. We identified, by screening a human kinase siRNA library, phosphatidylinositol 4-kinase type II (PI4KII) α and phosphatidylinositol-4- phosphate 5-kinase type I (PIP5KI) as required for Wnt3a-induced LRP6 phosphorylation at Ser1490 in mammalian cells and confirmed that these kinases are important for Wnt signaling in Xenopus embryos. Wnt3a stimulates the formation of phosphatidylinositol 4,5-bisphosphates [PtdIns (4,5)P2] through frizzled (Fz) and dishevelled (Dvl), the latter of which directly interacted with and activated PIP5KI. PtdIns (4,5)P2 in turn regulated phosphorylation of LRP6 at Thr1479 and Ser1490. Therefore, our study reveals a new signaling mechanism for Wnt to regulate LRP6 phosphorylation. (Science, Sep 5th, 2008)

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Biochemical Evidence for the Role of Heterotrimeric G Proteins in β-catenin/Wnt Signaling.

K. K. Jernigan, C. Cselenyi, E. Tahinci, E. Lee; Cell and Developmental Biology, Vanderbilt University, Nashville, TN

The Wnt receptor, Frizzled, contains seven predicted membrane-spanning domains characteristic of G-protein coupled receptors. Based on this observation, it has been proposed that G-proteins play a role in β-catenin/Wnt signaling. Previous evidence for the roles of Gαo and Gαq in the Wnt pathway has come primarily from studies using cultured mammalian cells and Drosophila genetics. Using Xenopus egg extracts, we now show that recombinant Gαo, Gαq, and Gβγ can inhibit β-catenin degradation when reconstituted into this biochemical system. Using the Topflash luciferase-system for Wnt target gene activation, we demonstrate that Gβγ acts synergistically with co-transfected LRP6 (Wnt co-receptor) in cultured mammalian cells. We further demonstrate by co-immunoprecipitation that Gβγ physically associates with Axin (a negative regulator of the Wnt pathway). We propose that Gβγ functions to promote membrane localization of Axin, thereby, enhancing the activity LRP6. In summary, these studies provide strong evidence for a critical role of Gβγ in β-catenin/Wnt signaling.

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Expression Profiles of Wnt Signaling Pathway Components in Response to cAMP Stimulation at Different Stages of Osteoblast Maturation.

R. Kao[1,2], W. Lu[1,2], A. Louie[1,2], R. Nissenson[1,2]; [1]University of California, San Francisco, San Francisco, CA, [2]Endocrine Unit, VA Medical Center, San Francisco, CA

Objective: The Wnt signaling pathway has been shown to play an important role in bone cell differentiation, proliferation, and apoptosis, therefore we sought to determine whether cAMP signaling in osteoblasts controls bone formation by regulating Wnt signaling differently at

different stages of osteoblast differentiation. Methods: Utilizing a real time RT-PCR based gene expression array, expression of 84 genes related to Wnt-mediated signal transduction was examined in 10-day (immature) and 23-day (mature) bone marrow derived murine osteoblast cultures. Cells were exposed to cAMP treatment for either a 3- or 24-hour duration at both differentiation time points to assess immediate-early and late gene regulation by cAMP. Results: Very little change in the expression of Wnt signaling genes was detected after 3-hours of cAMP treatment in 10-day and 23-day cultures. Treatment with cAMP for 24 hours induced changes in the expression of several Wnt signaling genes in both immature and mature osteoblasts. 24 hours of cAMP treatment down-regulated Wnt1 and Wnt11 expression by more than two-fold. Up-regulation of Wnt9a was only detected in 10-day cells, while down-regulation of Wnt2 and Wnt16 and up-regulation of Wnt4 were uniquely observed in the 23-day osteoblasts. Expression of a negative modulator of Wnt signaling, Sfrp4, was up-regulated by cAMP in 23-day osteoblasts. Additionally, we compared gene expression profiles of Wnt signaling components of 10-day and 23-day untreated osteoblasts to assess changes in Wnt signaling as cells differentiate. Dramatic changes in gene expression profiles were detected. Significant among these genes were Sfrp4, Wnt16, Wnt7b, and Wif1, which were up-regulated in mature cells. These results indicate that differentiation of osteoblasts is associated with increased potential to regulate Wnt signaling. The expression levels of several secreted modulators of Wnt signaling are altered as osteoblasts differentiate. On the other hand, relatively few Wnt-related genes appear to be subject to acute regulation by cAMP. Conclusions: Mature osteoblasts may represent an essential control point for the paracrine regulation of Wnt signaling in the bone marrow microenvironment.

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Cooperative and Distinct Roles of Wntless and the Retromer Complex in Wnt Signaling.

J. Han, H. Kim; Division of Molecular and Life Sciences, Pohang University of Science and Technology, Pohang, South Korea

Wnt signaling is implicated in a variety of developmental and pathological processes. The molecular mechanisms governing the secretion of Wnt ligands remain to be elucidated. Wntless, an evolutionarily conserved multi-pass transmembrane protein, is a dedicated secretion factor of Wnt proteins that participates in Drosophila embryogenesis. In this study, we show that Xenopus Wntless (XWntless) regulates the secretion of a specific Wnt ligand, XWnt4, and that this regulation is specifically required for eye development in Xenopus laevis. Moreover, the Retromer complex is required for XWntless recycling to regulate XWnt4-mediated eye development. Inhibition of Retromer function by Vps35 morpholino (MO) resulted in various Wnt deficiency phenotypes affecting mesoderm induction, gastrulation cell movements, neural induction, neural tube closure, and eye development. Overexpression of XWntless led to the rescue of Vps35 MO-mediated eye defects, but not other deficiencies. These results collectively suggest that XWntless and the Retromer complex are required for the efficient secretion of XWnt4, facilitating its role in Xenopus eye development. However, other Wnt deficiency phenotypes elicited by loss of the Retromer complex component, Vps35, are independent of Wntless recycling.

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Canonical Wnt Signaling in the Patterning of Feather Stem Cells.

C. Chiu[1], T. Jiang[1], M. Kahn[2], R. Widelitz[1], C. Chuong[1]; [1]Pathology, USC Keck School of Medicine, Los Angeles, CA, [2]Biochemistry and Molecular Biology, USC Keck School of Medicine, Los Angeles, CA

The formation of complex ectodermal organs begins with multipotent stem cells that undergo many basic cellular events. During the formation of a complex organ, there are many factors that need to be considered such as organization and relative size of each component in order to maintain proper organ function. Feather development is a good model. The number, size, and distribution of forming feather buds within a feather field must first be established. Canonical and non-canonical Wnt signaling has been implicated in many crucial steps in feather bud development. Canonical Wnt signaling involves the stabilization and accumulation of β-catenin, which is subsequently translocated to the nucleus. There, β-catenin interacts with various coactivators including CREB-binding protein (CBP) and p300, which results in the expression of different downstream genes to direct cells towards a path of pluripotency or differentiation. One of the genes downstream of β-catenin/p300 interaction is Myc. Myc activation has been shown to deplete epidermal stem cells by inducing their entrance into the transit-amplifying compartment followed by a few rounds of cell division before initiation of terminal differentiation along epidermal and sebaceous lineages. In vitro induction of Myc has also been found to be important in the regulation of cell growth and cell size. IQ and ICG, inhibitors of β-catenin interactions with p300 and CBP, respectively were used to perturb feather bud formation in embryonic chicken skin explant cultures grown in a SmartSlide environmental chamber (Wafergen, Fremont, CA). Time lapse video microscopy was used for analyses. We find that β-catenin interaction with p300 is indispensable for proper development of feather buds. Myc activation results in enlargement of forming feather buds.

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ADAM13 Is Required for Xenopus Head Development by Regulating Class B Ephrin and Wnt Signaling.

S. Wei[1], L. C. Bridges[1], P. Williams[1], G. Xu[1], C. A. Whittaker[2], A. Shah[1], S. O'Neill[1], J. M. White[1], D. DeSimone[1]; [1]Cell Biology, University of Virginia, Charlottesville, VA, [2]David H. Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA

Protein ectodomain shedding by disintegrin metalloproteinases (ADAMs) has emerged as a key regulatory event in cell signaling. We previously showed that an ADAM metalloproteinase, ADAM13, is expressed in cranial neural crest (CNC) cells in Xenopus laevis, and that overexpression of a protease dead mutant of ADAM13 prevents CNC migration. To further understand the roles of this gene in vertebrate development, we carried out loss-of-function studies in X. tropicalis, a closely related frog species with a diploid genome. Knockdown of ADAM13 by antisense morpholinos (MOs) resulted in abnormal head development, including reduced CNC induction and perturbed eye field patterning. The metalloproteinase activity of ADAM13 was required for these functions in vivo. Using cultured cells, we identified membrane-bound ephrins (Efns) B1 and B2 as substrates for ADAM13. Knockdown of ADAM13 protected endogenous EfnB1/B2 in developing embryos. The defects caused by ADAM13 MO could be rescued by blocking forward EfnB signaling, restoring canonical Wnt signaling, or by ectopic expression of the transcription factor Slug. Our data suggest a novel EfnB-Wnt-Slug signaling cascade through which ADAM13 controls CNC induction and eye field patterning.

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A Novel E3 Ubiquitin Ligase LNX-L Determines Dorso-ventral Body Patterning by Negatively Regulating Bozozok Stability.

H. Ro, I. Dawid; NICHD, NIH, Bethesda, MD

Ligand of Numb protein-X (LNX) is a RING finger and four PDZ domains containing E3 ubiquitin ligase. Although several reports suggested that LNX might serve as binding platform and have a role in cell fate determination, the in vivo functions of LNX remain unknown. Here we show that maternally transcribed LNX-L (LNX-Like), one of the zebrafish homologues of mammalian LNX, acts as a critical regulator of dorso-ventral axis formation. Depletion of LNX-L using specific antisense morpholinos (MO) caused strong embryonic dorsalization. Through the yeast two hybrid screening and ubiquitination assay, we identified that Bozozok (Boz) is a substrate of LNX-L. Boz is a homeobox containing transcription repressor whose expression is directly induced by canonical Wnt signaling, and is critical for the dorsal organizer formation. LNX-L induced K48 linked polyubiquitin chain outgrowth from Boz. The polyubiquitinated Boz was targeted to proteasomal degradation in human 293T cell line as well as zebrafish embryos. Embryonic dorsalization induced by boz overexpression was suppressed through addition of LNX-L in a dosage dependent manner. However, LNX-L failed to counteract embryonic dorsalization caused by overexpression of mutant Boz lacking LNX-L binding domain. In addition, the embryonic dorsalization induced by depletion of LNX-L was alleviated when boz expression was dampened. LNX-L is therefore acting as a limiting factor of Boz to prevent the invasion of dorsal tissue into the ventral region from Nieuwkoop center and dorsal organizer.

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A Critical Role of CSN Deneddylating Complex in Establishing the Hedgehog Signaling Gradient.

J. Wu[1], W. Lin[2], C. Chien[2]; [1]Department of Medical Research, National Taiwan University Hospital, Taipei, Taiwan, [2]Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan

Hedgehog (Hh) is a conserved morphogen that patterns various tissues including the Drosophila wing. In a developing Drosophila wing, Hh molecules are secreted from posterior compartment and diffuse across anterior compartment. Cells in anteroposterior boundary receive the highest Hh signaling which inhibits PKA, GSK3β and casein kinase-mediated phosphorylations of full length Cubitus interruptus (Ci^{155}). Lacking extensive phosphorylation, Ci^{155} escapes from the SCF^{Slimb}- mediated ubiquitylation and proteolysis. On the other hand, Ci^{155} in cells exposed to low levels of Hh is extensively phosphorylated by PKA, GSK3β and casein kinase and undergoes proteolysis. It is not clear how the SCF^{Slimb}-mediated proteolysis event coordinates with the Hh-mediated inhibition of Ci^{155} phosphorylation to achieve graded Ci^{155} proteolysis along anteroposterior axis in the anterior compartment of Drosophila wing. SCF^{Slimb} ligase activity is regulated by conjugating a Nedd8 moiety on Cullin 1 (Cul1). Nedd8 conjugation on Cul1 activates SCF^{Slimb} whereas removing Nedd8 moiety from Cul1 by the CSN complex inactivates SCF^{Slimb}. In *CSN* mutant cells, Ci^{155} proteolysis increases especially in cells receiving an intermediate level of Hh. In addition, expressing a dominant negative GSK3β abolishes Ci^{155} proteolysis in wild type cells but not in *CSN* mutant cells, indicating that the proteolysis-resistant, under-phosphorylated Ci^{155} is mistaken as a SCF^{Slimb} substrate in *CSN* mutant cells. As a result, *CSN* mutant cells receiving intermediate Hh signaling fail to activate Dpp expression. Taken together, we conclude that CSN coordinates SCF^{Slimb} E3 ligase activity with the graded Ci^{155} phosphorylation resulted form Hh signaling to achieve the graded Ci^{155} proteolysis. Therefore, deneddylation of SCF^{Slimb} is essential to translate the positional information laid down by external Hh gradient into the position-specific transcription programs in developing Drosophila wings.

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Determining the Biochemical and Cellular Interaction between Frazzled and Abelson Tyrosine Kinase.

B. Varughese[1], M. Brezden[1], M. Seeger[2], M. VanBerkum[1]; [1]Biological Sciences, Wayne State University, Detroit, MI, [2]Department of Molecular Genetics and Center for Molecular Neurobiology, Ohio State University, Columbus, OH

Frazzled is a chemoattractive receptor required for directional movement of axons across the midline during development of the Drosophila embryonic nerve cord. Frazzled regulates cytoskeletal dynamics via multiple downstream effectors acting through three conserved P-motifs in its cytoplasmic domain. A cytoplasmic tyrosine kinase, Abelson Kinase (Abl) is a key regulator of actin dynamics and genetically interacts with Frazzled to help guide commissural axons across the midline. Too much and too little Abl interact with Fra to prevent commissure formation. Abl also interacts biochemically with the cytoplasmic domain of Frazzled and here, using Drosophila S2 cells, we demonstrate that Abl binds Frazzled independent of any of the three P- motifs. Similarly, a hyperactivated form of Abl (BcrAbl) and kinase inactive forms (AblKN and BcrAblKR) also physically interact with Frazzled. Ectopic expression of Frazzled in S2 cells produces very long filopodia-like extensions in over 70% of the cells when plated on concanavalin A coated cover slips. Frazzled is localized to the tips of these phalloidin stained processes. Filopodia are reduced when either the P1 or P3 motif is removed. Co-expression of Frazzled and wild type Abl or especially BcrAbl in S2 cells inhibits the length of the filopodial extensions and instead numerous microspikes appear. The microspikes are evident when only Abl or BcrAbl are expressed in S2 cells but in addition, these cells exhibit membrane ruffling. Expression of a kinase inactive form of BcrAbl also inhibits the formation of Frazzled-dependent filopodia and instead cells become enlarged and spindle shaped; microspikes are absent. Thus, while both Frazzled and Abelson appear to regulate aspects of actin dynamics, Frazzled activity induces long filopodia while Abl activity preferentially induces microspikes. This suggests that, in the developing embryonic nerve cord, Frazzled and Abl may operate in parallel pathways to regulate actin dynamics. This work is supported by the National Science Foundation.

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Deciphering T-box1 Pathway in an In Vitro Model.

K. K. Yee[1,2], H. Yagi[1], R. Matsuoka[1]; [1]International Research and Educational Institute for Integrated Medical Sciences (IREIIMS), Tokyo Women's Medical University, Tokyo, Japan, [2]Division of Advanced Biomedical Engineering and Science, Tokyo Women's Medical University, Tokyo, Japan

Objectives: In understanding T-box1 (Tbx1) associated molecular pathways could potentially help us to comprehend varied phenotypic expression of DiGeorge (DGS)/Conotruncal anomaly face syndrome (CAFS); also known as Velo-cardio-facial syndrome (VCFS). Methods: P19, an undifferentiated pluripotent embryonal carcinoma cell line generated from the testis of C3H/He mouse was used in the experiments.

Tbx1 short hairpin interference RNA was generated with pSilencer Hygyro (Ambion). Subtractive hybridization kit (Clonetech) was used to distinguish genes that are expressed in the presence and absence of Tbx1. Results: Tbx1 gene was identified previously as one of the causative gene that influences the incidence of DGS/CAFS. Hence Tbx1 knockdown was used to identify the potential pathways of Tbx1. The subtractive hybridization study showed that 125 genes were identified out of 265 clones analyzed. Forty-five percent of these genes were involved in cell cycle/ growth function. Twenty-one genes were randomly selected and PCR to determine if there was a difference in their expression at RNA level. The data indicated that 10 genes showed a significant differential gene expression between control and Tbx1 knockdown sample. Conclusion: Subtractive hybridization data shows Tbx1 regulates genes involved in cell cycle, development and energy metabolism. This data could potentially expand the current knowledge on Tbx1 molecular pathways.

604/B566

The Role of Stromal Derived Factor-1α in Vertebrate Somitogenesis.

M. A. Leal; Department of Biology, San Francisco State University, San Francisco, CA

Stromal derived factor-1α (Sdf-1α), a chemoattractant chemokine, along with its exclusive receptor CXCR4, plays a major role in tumor growth, angiogenesis, metastasis and in development. Recently, the Sdf-1α signaling pathway was shown to be important for zebrafish somitogenesis (Holloway, et al 2007). Somitogenesis is an important embryological process as it establishes the segmented nature of the vertebrate body plan as well as giving rise to the adult skeletal and musculature system. During zebrafish somitogenesis, cells within the somite undergo rotation prior to myogenesis. This rotation behavior requires Sdf-1α signaling. Interestingly, somite cell rotation also occurs in the frog, Xenopus laevis; however the molecular mechanism underlying this process remains unknown. Therefore, we examined whether Sdf-1α signalling is also required for somite rotation in Xenopus laevis. Our approach was to inject morpholino antisense oligomers into one of two blastomeres at the two-cell stage to knock down the expression of Sdf-1α and its receptor, CXCR4 during somitogenesis. Injected embryos were then fixed and stained with the myotome-specific antibody, 12/101. The embryos were then imaged with a confocal microscope. We found that a knock down of Sdf-1α caused a range of phenotypes from a complete block of somite formation to the misalignment of myotome fibers. Similarly, a knockdown of CXCR4 also led to a disruption in axis elongation and somite formation. These results suggest that Sdf-1α signaling is important in directing the cell movements during somite formation in Xenopus, and that this pathway is conserved between fish and frogs.

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Roles of Tryptophan Hydroxylase in Early Development.

S. Maegawa[1,2], Y. Sawada[1], H. Hosokawa[1], E. S. Weinberg[2], S. Kobayashi[1]; [1]Department of Intelligence Science and Technology, Kyoto University, Kyoto, Japan, [2]Department of Biology, University of Pennsylvania, Philadelphia, PA

Tryptophan hydroxylase (TPH) is a rate-limiting enzyme in serotonin synthesis. We found that one of the tryptohan hydoxylase genes in zebrafish (tph1b) was expressed in the spemann organizer in zebrafish embryos. The fact led us to examine whether TPH and/or serotonin is involved in early developmental processes. To examine roles of Tph1b in early development, we examined effects of p-Chlorophenylalanine (PCPA), a strong inhibitor for TPH. As the results, PCPA-exposure from 8-cell stage shortened the body axis. To understand the phenotype, we first examined the dorsoventral polarity in the treated embryos. The results clearly indicated that there was no obvious defect in the dorsoventral patterning during gastrulation. In contrast, the PCPA-treated embryos clearly showed expanded neural plate at 3-somite stage, suggesting that PCPA disturbs cell movements rather than cell fate determination. We obtained essentially same results when the morpholino antisense for tph1b was injected. Taken together, we conclude that Tph1b is involved in the specific cell movements during early development such as convergence and/or extension.

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Changes in mAKAP and PDE4D3 Expression during L6 Myogenic Differentiation.

S. K. Byrd, L. Clah, L. Lowder, S. Moon, B. Walters; Biology, Fort Lewis College, Durango, CO

The role of A Kinase Anchoring Protein (AKAP) in the development of L-6 myoblasts is not fully understood. We measured the expression of the muscle-specific mAKAP through the differentiation process. Due to the intimate relationship of this dual role AKAP and the cAMP pathway, we also measured the cAMP levels via ELISA and phosphodiesterase type 4 isoform D3 (PDE4D3) expression. mAKAP and PDE4D3 were measured relative to β-actin via RT-PCR under normal conditions and siRNA transfections with the RNAi for mAKAP. Myosin heavy chain (MHC) and mAKAP protein concentrations were determined relative to GAPDH using Western blotting; no MHC was expressed in 3 day myoblasts, whereas 9 day myotubes expressed 36% myosin relative to GAPDH. mAKAP protien band intensity as a percent of GAPDH was higher in blasts (30%) than in tubes (20%). mAKAP mRNA message as a percent of β-actin was similar in blasts (43.6%) and in tubes (41.0%). MHC mRNA band intensity as a percent of β-actin was 8.0% in tubes and not present in blasts (0.0%). PDE4D3 48.0% in blasts and 18.5% in tubes, relative to β-actin. There was also significant change in overall AKAP composition during development. There was no significant change in [cAMP] in 3-day myoblast versus 9-day myotubes (0.00072 pmol/ug and 0.0122 pmol/μg protein, respectively). mAKAP appears to remain constant throughout development, but one of its partners, PDE4D3 does not appear to be reduced as the differentiation process is completed.

607/B569

Myotome Development Regulated by Membrane Rafts and a Constituent Nitric Oxide Synthase in Chicken Embryos.

W. F. Denetclaw, S. Lee; Biology, San Francisco State University, San Francisco, CA

Embryo development is regulated by messenger molecules like nitric oxide (NO) and intracellular free calcium (Ca2+). Constitutive nitric oxide synthase (NOS) isoforms require Ca2+-calmodulin binding to produce NO. In addition, constitutive NOS isoforms associate with multifunctional membrane rafts (rafts). We report an abundance of rafts in ectoderm, somite and neural tube of chicken embryos although their role in embryo development is not understood. Because the ectoderm regulates myoD expression, a muscle regulatory gene, ectoderm rafts may effect molecular signaling to the somite for control of myotome formation. To test this, we disrupted ectoderm rafts by a brief 6

hour treatments with methyl-beta-cyclodextrin (MBC) and blocked the formation of nascent myotome in somites (stages VII-X). To investigate NO signaling between ectoderm and somites, NO specific fluorescent probes (DAF-2, DAR-4M) was used to label embryo tissue sections made through somites and adjacent tissues and NO levels monitored by confocal microscopy. Our results show for the first time that the ectoderm initiates a large and dynamic increase in NO that later causes a second large and dynamic rise in NO in somites. The rise in NO was limited to the dorsal epithelial cell layer of nascent somites and to the myotome forming dermomyotome layer in older somites. In ectoderm, a few isolated cells in the outer-most periderm layer initiated the rise in NO before spreading to large numbers of other adjacent periderm and sub-periderm cells in ectoderm to suggest Ca2+ activation of NOS and movement across the epithelium by gap junction coupling. We now report that Ca2+ precedes NO elevation in the ectoderm and somite to show that constitutive NOS isoforms are responsible for increasing NO. We conclude that raft-associated constitutive NOS isoforms in the ectoderm regulate NO levels for subsequent signaling to somites for myotome formation. NIH-RIMI-P20MD000544; NSF-IOS-0821324.

608/B570

Identifying Molecules Involved in the Signal Transduction Pathway at Bovine Oocyte Activation Using Activators and Inhibitors of Src Family Kinases.

A. H. Bayles, J. Collier, A. P. Davis, B. R. Sessions, K. L. White; Animal, Dairy and Veterinary Sciences and Center for Integrated Biosystems, Utah State University, Logan, UT

Intracellular calcium (Ca^{2+}_i) release, a hallmark of oocyte activation, is the end result of a complex series of signal transduction pathways which have yet to be completely characterized in the bovine model. It is well known that src family kinases (SFK) activate Phospholipase C (PLC) which converts phosphatidyl inositol (4,5)-bisphosphate into diacylglycerol and 1,4,5-inositol trisphosphate (IP_3) which is directly involved in releasing Ca^{2+}_i from the endoplasmic reticulum. A two prong approach was taken to identify the mechanisms involved in the signal transduction pathway which results in bovine activation. The first approach utilized immunoblotting to determine the presence of endogenous PLC isoforms and SFKs in total bovine oocyte lysate by using primary antibodies against the five distinct isoforms of PLC as well as primary antibodies against several SFKs. Immunoblotting was performed according to standard laboratory protocols and confirmed the presence of several PLC isoforms as well as some members of the SFKs. The second approach involved the microinjection of known inhibitors/activators specific to SFK signaling cascades. A range of concentrations for each inhibitor/activator was microinjected in addition to a sham injection to assist in evaluating oocyte quality. Following microinjection, oocytes were fertilized and cultured in vitro according to standard laboratory procedures (Reed et al., 1996). Activation and development were assessed by recording cleavage at 48 hours post fertilization. Microinjection of several inhibitors resulted in no effect on cleavage rates while other inhibitors essentially blocked development. Determining the presence and involvement of these intracellular signaling molecules in bovine oocyte fertilization and activation will result in better activation protocols for nuclear transfer (NT) embryos and ultimately improve the efficiency of the NT process along with facilitating study of specific aspects of activation by identifying messages associated with the process.

609/B571

Dictyostelium B56 Is Essential for Both Differentiation and Cell Migration.

M. Rodriguez, N. Lee, S. Veeranki, B. Kim, L. Kim; Florida International University, Miami, FL

B56 is a well-known regulatory subunit of PP2A in metazoan development. We have uncovered that B56 also plays essential roles in non-metazoan development. *Dictyostelium* b56-null cells (*psrA*-) were generated by inserting a *Blasticidin* cassette by homologous recombination in one of the two PP2A subunit A binding domains (ASBDs) present in B56(DB56). This protein plays an inhibitory role in PP2A regulation, since ablation of *DB56* resulted in a higher PP2A activity. Upon starvation, *psrA*- cells showed delayed development. At the molecular level, prestalk markers, ecmAO and ecmB and prespore marker cotB, had aberrant expression patterns compared to wild type cells. In addition, *psrA*- cells showed higher levels of GSK3 activity. Alleviation of higher GSK3 activity by dominant negative GSK3 restored prestalk and prespore cell differentiation similarly to that of wild type cells. These results indicate B56 plays a role in cell fate determination during development. In addition, *psrA*- cells failed to properly activate the GTPase Ras in response to cyclic AMP (cAMP) stimulation. Furthermore, cAMP-pulsed *psrA*- cells showed compromised random and directional motility. We will present these novel functions of B56 in *Dictyostelium* cells.

610/B572

Copine A Is Necessary for the Production/Release of a Signaling Factor that Triggers Spore Cell Differentiation in Dictyostelium Development.

T. S. Smith, J. M. Pineda, K. A. Lepley, C. K. Damer; Biology, Central Michigan University, Mount Pleasant, MI

Copines are calcium-dependent membrane-binding proteins found in numerous eukaryotic organisms. We are studying the function of copine A (*cpnA*) in *Dictyostelium* development. When placed in starvation buffer, wildtype (WT) *Dictyostelium* cells develop into fruiting bodies consisting of spores atop a stalk. In cells lacking copine A (KO), development becomes arrested; KO cells form finger-like structures or structures that look like slugs on top of stalks. Time-lapse imaging revealed that KO cells aggregated into mounds with normal timing, but formed larger mounds than WT cells. The KO mounds then developed into larger slugs that were less motile than the smaller WT slugs. Mixing KO cells with a small % of WT cells expressing green fluorescent protein (GFP) rescued the developmental defect. At 5% WT cells, fruiting bodies with short stalks formed. As the % of WT cells was increased, the stalks became longer and reached normal length at 50% WT cells. Spores within individual fruiting bodies contained both cell types at the same original mixing ratio. When a small % of KO cells expressing GFP were mixed with WT cells, the KO cells were found throughout the developing structures and did not preferentially differentiate into spore or stalk cells. These data suggest that KO cells are not able to release a signaling factor necessary for spore cell differentiation, but they are able to respond to the factor released from the WT cells. We next developed both WT and KO cells on filters placed on pads soaked in buffer. At various times in development, we replaced the WT filters with the KO filters. When filters were switched at late stages of development, fruiting bodies formed on the KO filters indicating that the KO cells were responding to a diffusible factor released by WT cells. To study the calcium regulation of *cpnA*, KO cells were developed in buffer with various calcium concentrations. In

reduced calcium, KO cells formed fruiting bodies with short stalks suggesting that *cpnA*'s regulation by calcium is important to its role in development. Our results suggest that *cpnA* has a role in the release or production of a diffusible factor late in development necessary for the differentiation of spores.

611/B573

RNA Helicases Are Important for Plasmodesmal Development and Function.

T. Burch-Smith, P. Zambryski; Plant & Microbial Biology, University of California, Berkeley, Berekely, CA

In plants, intercellular transport and communication across their ubiquitous cell walls is facilitated by plasma membrane-lined, cytoplasmic channels called plasmodesmata (PD). PD serve as conduits for trafficking of developmentally important macromolecules including transcription factors. Further, plant pathogens, particularly viruses, subvert PD to accomplish cell-to-cell spread during infection. Our lab conducted a genetic screen to identify genes that are required for PD function, as demonstrated by their enhanced transport of fluorescently labeled probes. This screen identified several *Arabidopsis thaliana* genes, that when mutated, result in embryonic lethality. These mutations reveal the importance of PD function in plant development. We have mapped and cloned two of these genes: *INCREASED SIZE EXCLUSION LIMIT (ISE)1* and *ISE2*; both encoding distinct RNA helicases. Interestingly, loss of ISE2 results in dramatic alterations to PD morphology in *A. thaliana* embryos. Our aim was to examine the role of these RNA helicases in intercellular transport and general development. For this, we identified *Nicotiana benthamiana* homologs and silenced them in *N. benthamiana* using Tobacco rattle virus-based Virus induced gene silencing (VIGS). This approach allows us to study the function of these essential genes in adult tissues. *ISE1* and/or *ISE2*-silenced plants exhibit striking yellowing of silenced tissues. The onset, pattern and duration of the variegation are different between *ISE1* and *ISE2*-silenced tissues, suggesting different functional roles for these two helicases. Note, silenced plants show no other changes in their vegetative or reproductive development. Importantly, plants with reduced levels of *ISE1* or *ISE2* transcript show altered timing of systemic spread of Tobacco mosaic virus and cell-to-cell transport of its movement protein, MP30. Further, transmission electron microscopic analysis reveals changes in PD distribution and structure in young, silenced leaves. These studies reveal that in addition to essential roles in embryogenesis, ISE1 and ISE2 are also critical factors regulating PD development and function in mature tissues.

612/B574

Growth Determination of the Inflorescence Is Influenced by IGI1 in Arabidopsis.

I. Hwang, Y. Yang, H. Cheong; Chosun University, Gwangju, South Korea

The phenotypically distinct plants were screened in the activation tagged lines and a mutant, igi1 (Inflorescence Growth Inhibitor 1) was isolated. Segregation ratio of the F2 generation, TAIL-PCR walking and genotyping PCR results indicated a single T-DNA insertion at the 200bp upstream of the At1g23540 coding region in the BAC F5O8. We confirmed At1g23540 gene over-expression by Real time PCR. The homozygous mutant showed sterile, no inflorescence, no seeds and abnormal flower organ after the plants start to flower. The heterozygous mutant plants produced primary inflorescence with reduced internode elongation, and secondary inflorescences begin to emerge straight and also, dramatically increased axillary branching. The igi1/igi1-RNAi reverted to wild type phenotype by reducing the mRNA levels. The transcript level of auxin biosynthesis component, CYP79B2 was increased in the igi1 mutant. In the cell division and callus greening test for cytokinin response, igi1 showed similar pattern. When grown in dark, wild type and igi1/igi1 mutant showed similar patterns in hypocotyl growth and in 2,4-D and kinetin dose response. Interestingly, the root growth showed inhibition pattern in igi1/igi1 mutant under light condition (0nM hormone concentration) and the relative deference in root growth between wild type and igi1/igi1 mutant diminished in BAP dose response. In the medium supplemented with 2,4-D, the growth showed no response. These results indicated that there is noble pathway of the axillary meristem development through IGI1, which was modulated by light regulated pathway.

613/B575

The Role of BRCA1 in ATM-mediated PP1 Activation after DNA Damage.

X. Tang, B. Xu; Department of Biochemistry and Molecular Biology, Southern Research Institute, Birmingham, AL

Optimal DNA damage responses are controlled by a comprehensive signaling network which consists of damage sensors, transducers, effectors and adaptors. Among them, the ATM kinase is considered a critical element in the signaling pathways. Upon DNA damage, ATM binds strongly to damaged sites and its kinase activity is enhanced. Activated ATM, in turn, phosphorylates a list of substrates in pathways that together ensure cellular survival and recovery. We have reported that ATM regulates Protein Phosphatase 1 (PP1) activity in response to DNA damage by phosphorylation of Inhibitor-2 (I-2), a regulatory subunit of PP1. However, the detailed mechanism of the signaling network involving ATM, PP and I-2 has not been elucidated. In the present study, we found that BRCA1, a protein important for DNA repair and cell cycle checkpoints, is required for ATM-mediated I-2 phosphorylation and PP1 activation after DNA damage. Furthermore, we find BRCA1 is essential for recruiting I-2 to DNA damage sites, and BRCA1 interaction with I-2 increases after DNA damage. This DNA damage-induced BRCA1/I-2 interaction further lead to I-2/PP1 dissociation, the process required for PP1 activation after DNA damage. Taken together, our results demonstrate that BRCA1 plays an essential role in PP1 activation as a signal adaptor between ATM and I-2.

Stem Cells I (614-635)

614/B576

Analysis of the SAGE Expression Profiles of Human Embryonic Stem Cells Identifies a Robust Set of Candidate Genes for Pluripotency and Differentiation.

Z. Lai[1], V. Shubha[1], R. Kumar[2], Y. Wang[1], M. Richards[1,2], A. Bongso[2], W. Chan[1]; [1]Department of Biological Sciences, National University of Singapore, Singapore, Singapore, [2]Department of Obstetrics and Gynecology, National University of Singapore, National University Hospital, Singapore, Singapore

A plethora of research has focused on the human embryonic stem cells (hESC) ever since they were first reported mainly due to their distinct features of self renewal and pluripotency. Probing of the hESC transcriptome using global expression profiling tools such as DNA microarray, Serial Analysis of Gene Expression (SAGE), Massively Parallel Signature Sequencing (MPSS) and Expressed Sequence Tag (EST) analysis have contributed significantly in our current understanding of hESCs. However, till date clarity is lacking in identifying a robust set of markers to assess the true state of the hESCs. In this work, we report the generation of long SAGE libraries for partially differentiated and differentiated HES3 along with a deeper profiling of our previously reported HES3 undifferentiated library. Clustering analysis of these libraries in concert with long and short SAGE libraries available in public databases using Hierarchical Cluster Analysis (HCA) as well as a poison-based approach helped in identification of expression patterns distinct from those reported for the well established pluripotency/differentiation markers. Almost all the previous studies reporting a robust set of markers have focused on a gene by gene comparison in listing out the upregulated and downregulated genes rather than look at the expression patterns in establishing a list of markers. In this analysis, however, we report a new set of markers as well as add confidence to some previously reported markers based on their novel expression patterns as identified by SAGE analysis and also confirm it by real-time PCR analysis. For the real-time PCR confirmation, instead of taking two extreme data sets such as undifferentiated and a late stage embryoid body, we profiled a time series of embryoid body stages so that we could identify those genes which show a dramatic increase or decrease upon differentiation and hence would serve as more reliable markers.

615/B577

Metabolic Analysis of Pluripotent vs. Differentiating Murine ES Cells.

D. Larsen, L. Rickords, J. Jones; Utah State University, Logan, UT

Much research has already been performed utilizing genomics and transcriptomics in determining which genes are involved in regulating pluripotency of Embryonic Stem (ES) cells. The objective of this experiment was to compare the metabolic profiles of murine pluripotent ES cells grown on murine embryonic fibroblasts (MEF) and differentiating murine ES cells grown on gelatin. Both treatment groups were cultured in Knockout DMEM (Invitrogen) supplemented with 15% Knockout Serum Replacement (Invitrogen), and 5% D2O (heavy water, Cambridge Isotope Laboratories, Inc.) to serve as a metabolic biomarker. Both treatment groups were then put through a derivitization process in order to isolate the various metabolites present in each sample. Briefly, each sample was suspended in cold 100% methanol, after which cellular metabolites were extracted using an equivalent volume of chloroform. Aqueous and organic layers were then separated and dried. Dry extracts were then derivitized using methoxyamine hydrochloride and pyridine, followed by N-Methyl-N-TMS-Trifluoroacetamide (MSTFA). Samples were dried once again and resuspended in a pyridine/MSTFA solution. Samples were then subjected to Gas Chromatography/ Mass Spectrometry (GC/MS) analysis for metabolite identification, with samples of MEF cells and fresh medium as metabolite controls. Our results indicate critical differences in metabolite concentrations between pluripotent and differentiating ES cells. Data will be presented illustrating specific metabolite profiles of pluripotent murine ES cells compared to differentiating ES cells. These Data will assist in better understanding the metabolic characteristics of differentiating ES cells, thereby allowing us to determine the state of differentiation through metabolic analysis.

616/B578

Mitochondrial Properties as an Indicator of Embryonic Stem Cell Competence.

T. Lonergan[1], B. Bavister[2], A. Harvey[3], C. Brenner[4]; [1]Department of Biological and Environmental Sciences, Western Connecticut State University, Danbury, CT, [2]Department of Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, MI, [3]Department of Physiology, Wayne State University School of Medicine, Detroit, MI, [4]Departments of Physiology and Obstetrics and Gynecology, Wayne State University School of Medicine, Detroit, MI

A previous study has suggested that adult stem cell mitochondrial characteristics are a potential predictor of stem cell competence (Lonergan et al., 2006). The aim of this study was to characterize mitochondrial properties in ES cells of two species to determine whether mitochondrial function may be used as an indicator of ES pluripotency and self renewal. Three rhesus macaque ES cell lines (ORMES 6, 7 and 22) and one mouse ES cell line (E14Tg2A) displayed a considerably large mitochondrial population as determined by staining with Mitotracker red. For the three monkey ES cell lines, the mitochondria were clustered next to one side of the nucleus (a morphology we termed “capped”) in 92-98% of the cells. Consistent with a previously reported monkey adult stem cell line, ORMES 7 had a significantly lower ATP content/cell in comparison to a monkey fibroblast line (7.55 +/- 0.95 (SEM) pmoles ATP/cell vs. 12.38 +/- 0.14 (SEM) pmoles ATP/cell, P = 0.02). The localization of Polymerase gamma (PolG) and Mitochondrial Transcription Factor F (TFAM), factors regulating mitochondrial DNA replication and transcription, respectively, were examined by immunofluorescence. In all ES cell lines, both PolG and TFAM co-localized in the mitochondria, suggesting that the mitochondria were competent to replicate. In contrast to the monkey ES lines, the mouse E14 line did not show a predominantly capped morphology and an ATP content/cell statistically identical to a fibroblast control. These data suggest that the mitochondrial cellular location pattern and the low ATP content/cell may be indicators of competence in primate ES cells, but not in mouse.

617/B579

Synergistic Effects of BIO, Activin A and BMP4 on Mesodermal Induction of Human Embryonic Stem Cells.

T. N. Tran, S. Park, E. Lee, Y. Han; Biological Sciences, KAIST, Daejeon, South Korea

A variety of intracellular signaling pathways are involved in the self-renewal and differentiation of human embryonic stem cells (hESCs). This study was performed to test the functions of TGF-β, WNT, and BMP signaling pathways in the hESC differentiation. hESCs were treated with Activin A, BMP4, and GSK3-inhibitor (BIO-6-bromoindirubin-3'-oxime) in feeder-free condition, and then expression of lineages-specific genes were checked at mRNA and protein levels. As results, the treatment of BIO, BMP4 or Activin A alone could not or slightly induce the expression of mesoderm marker genes such as T, Goosecoid, MIXL1, FLK1, and WNT3 and so on. Transcripts of mesoderm marker genes were enriched when hESCs were cultured in the medium containing combination of BIO+Activin A and BIO+BMP4, respectively. An experimental group treated with BIO+Activin A+BMP4 showed a higher expression level of mesoderm marker

genes than the other experimental groups, In addition, signaling inhibitors such as SB431542, DKK1 and Noggin significantly down-regulated the expression of mesoderm maker genes in hESCs. In conclusion, these results indicate that combined effect of BIO, BMP4, and Activin A may specify hESCs into mesoderm progenitor cells. These findings provide a new efficient method to induce mesoderm progenitor cells from human embryonic stem cells.

618/B580

Defining of Sox2 Functional Target Genes in ES Cells and Preimplantation Embryos.

C. Wang[1], L. Sun[1], C. Woon[1], P. Robson[1,2]; [1]Genome Institute of Singapore, Singapore, [2]Biology Sciences, National University of Singapore, Singapore

Sox2 is a high mobility group (HMG) DNA binding domain transcription factor that is essential for both mouse early development and for the maintainance of pluripotent embryonic stem (ES) cells. From genome-wide studies it is clear that Sox2 binds in close proximity to hundreds of genes in the ES cell genome. In addition to a sox cis element, many of these binding sites contain an octamer element, emphasizing Sox2 co-regulation of target genes with Oct4. However, the molecular mechanisms of Sox2 action has yet to be fully characterized, particularly in the preimplantation embryo, in part due to the presence of maternal Sox2 and redundancy with other Sox genes. To begin to address these issues we have resorted to using chimeric contructs containing the Sox2 HMG domain fused either to the potent transactivator VP16 domain (Sox2-VP16) or to the potent engrailed repressor domain (Sox2-EnR). BeadArray expression analyses of ES cells over-expressing these fusion constructs aid in determining a function of the many previously identified Sox2 binding sites. Genes both with associated Sox2 binding sites and showing chimeric construct-induced altered ES cell expression were classified as functional direct targets of Sox2. Interestingly, this also revealed a significant number of Sox2 target genes that are independent of Oct4 binding. To determine if these Sox2 functional direct targets were also regulated in preimplantation development we examined gene expression by real time PCR in mouse embryos transduced with lentiviruses expressing the Sox2-VP16 or Sox2-EnR fusion proteins. This data provides molecular insight into the cause of early lethality of Sox2 null embryos and into the architecture of the preimplantation genetic regulatory network.

619/B581

The Rho-Rock-Myosin Signaling Axis Determines Cell-Cell Integrity of Self-renewing Pluripotent Stem Cells.

N. Harb[1], T. K. Archer[2], N. Sato[1]; [1]Biochemistry, University of California Riverside, Riverside, CA, [2]Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Research Triangle Park, NC

Embryonic stem (ES) cells self-renew as coherent colonies in which cells maintain tight cell-cell contact. Although intercellular communications are essential to establish the basis of cell-specific identity, molecular mechanisms underlying intrinsic cell-cell interactions in ES cells at the signaling level remain unexplored. Here we show that endogenous Rho signaling is required for the maintenance of cell-cell contacts in ES cells. Rock, a major effector downstream of Rho, regulated myosin II by controlling a myosin light chain phosphatase. Chemical engineering of this signaling axis by a Rock-specific inhibitor revealed that cell-cell adhesion was reversibly controllable and, unexpectedly, dispensable for self-renewal of mouse ES cells confirmed by chimera assay. Furthermore, a novel culture system combining a single synthetic matrix, defined medium, and the Rock inhibitor fully warranted human ES cell self-renewal independent of animal-derived coatings, tight cell contacts, or fibroblastic niche-forming cells determined by teratoma formation assay. These findings provide a new insight into understanding basic cell-cell communication mechanisms and self-renewal of ES cells, and contribute to advance in medically compatible xeno-free environments for human pluripotent stem cells.

620/B582

Characterization of Stem Cells from the Umbilical Cord at Maternal and Fetal Ends.

J. J. Nance[1], R. Schugar[1], B. M. Deasy[1,2]; [1]Stem Cell Research Center, Children's Hospital of Pittsburgh, Pittsburgh, PA, [2]Orthopaedics and Bioengineering, University of Pittsburgh, Pittsburgh, PA

Mesenchymal stem cells(MSCs) from the human umbilical cord(UC) come from an inexhaustible source and offer an alternative to bone marrow. Although UC blood is well characterized and currently used clinically for blood transplantations, the UC tissue lacks such characterization and is routinely discarded after birth or blood harvest. We recently isolated cells from the umbilical cord and showed their potential as candidates for cartilage repair cell-therapy. To optimize cell isolation methods, regional in situ differences of cell phenotype and density were tested throughout the tissue. Methods: Immunohistochemical analysis for MSC markers CD44, CD73, CD90 and CD105 was used on full-term UC's (n=3), segmented into 2cm samples, gel-mounted, flash frozen and sectioned into 10µm slide cross-sections. Investigation was initiated at the ends with 3 regions of interest:Wharton's jelly(WJ), perivascular regions(PV), and blood vessels(V). Results: Results suggest that UC tissue from maternal(M) or fetal(F) ends yield little significant difference in MSC expression, although variations between the individual marker's was observed. Histologically, variation of regional cellular density was observed. We found overall low expression of CD44 in all areas of the UC- WJ, PV, and V. CD73 expression was ~100% in the vessels, absent in PV, and moderate in WJ. CD90 had similar expression in all regions; high levels were observed in WJ (86% M, 92% F), PV (91% M, 87% F), and V (~100%). CD105, showed no significant difference in expression between ends in all regions. Cellular density observation showed no significant difference in WJ or PV, whereas the vessels had the highest cellular density and differed significantly(p=0.011). Conclusions: Differential MSC expression along the UC provides another facet of variation to this tissue's characterization. We do observe differences in cell density throughout the cord length, which may affect cell harvest yields. However, to date, regional differences of cell phenotype have not been identified. In sum, these findings support current methods of cell harvest which show high yield for UC-MSC, capable of participating in cartilage tissue engineering and cell-mediated gene therapeutics.

621/B583

Characterization of Human Dental Pulp Stem Cells Isolated from Impacted Third Molars.

J. Karbanova[1,4], T. Soukup[1], J. Suchanek[2], L. Kucerova[3], D. Corbeil[4], J. Mokry[1]; [1]Department of Histology and Embryology, Charles University in Prague, Faculty of Medicine in Hradec Kralove, Hradec Kralove, Czech Republic, [2]Department of Dentistry, Teaching Hospital

in Hradec Kralove, Hradec Kralove, Czech Republic, [3]Department of Clinical Genetics, Teaching Hospital in Hradec Kralove, Hradec Kralove, Czech Republic, [4]Tissue Engineering Laboratory, BIOTEC, Technical University of Dresden, Dresden, Germany

In the present study, we isolated stem cells (SCs) from dental pulp of extracted third molars using innovative culture method of serum-low-content medium supplemented with growth factors PDGF-BB and EGF. We further evaluated differentiation potential of dental pulp stem cells (DPSCs) cultured adherently or as micromass/spheroid cultures in various defined media. Phenotype of undifferentiated and differentiated cells was analyzed by flow cytometry, Western blot and immunocytochemistry. Under our culture conditions isolated DPSCs were expandable over the 60 population doublings and during the entire culture period they maintained stable karyotype. DPSCs were characterized as a cell population that was STRO-1$^+$, CD29$^+$, CD44$^+$, CD90$^+$, HLA I$^+$, CD49a,c,d,e^{low}, CD11b,c^{low}, CD117^{low}, CD34$^-$, CD45$^-$ and HLA II$^-$. Immunocytochemical analysis confirmed expression of stem cell associated markers including hTERT, CXCR4, nanog, nucleostemin, nestin, β1-integrin. They were weakly positive for VEGFR2, Bcrp1 and MDR1 and negative for neural, vascular and hepatic cell markers. When placed in standard osteogenic media DPSCs were found to differentiate in osteonectin/procollagen type I$^+$ osteoblasts. In micromass cultures cells formed osteogenic pellets rich in collagen with depositions of calcium. Likewise chondroblasts were generated in chondrogenic pellets in TGF-β1 supplemented media and produced extracellular matrix typical for cartilaginous tissue. The addition of VEGF to serum-free media resulted in expression of endothelial markers vWF, CD31, CD34, CD105 and CD106. When the DPSCs were placed in medium for neural SCs supplemented with serum cells exhibited markers of immature neural cells (nestin, A2B5, NG2, RC2, FORSE-1). Exclusion of serum from media facilitated cell differentiation and expression of neuronal and glial markers. Taken together our results demonstrate that cells isolated from dental pulp are self-renewing, cytogenetically stable and able to generate bone, cartilage, vascular and neural cells, indicating that they are multipotent. Their easy accessibility as well as the broad differentiation capacity make these cells a suitable alternative source of SCs for tissue engineering. Supported by GACR 304/07/P307.

622/B584

Quantitative Analysis of Therapeutic Muscle Cell Populations through Live Automated Cell Imaging.

S. Chirieleison[1,3], C. Scelfo[1,3], B. M. Deasy[2,3]; [1]Stem Cell Research Center, Children's Hospital of Pittsburgh, Pittsburgh, PA, [2]Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA, [3]McGowan Institute of Regenerative Medicine, University of Pittsburgh, Pittsburgh, PA

Visible image analysis is attractive for distinction of heterogeneous therapeutic cell populations for which traditional methods offer limited insight. Current visible image analysis for cell morphological and behavioral data is subject to a number of set backs including availability of automated analysis software, limited parameter inclusion, and subjectivity. We describe and present here a morphological analysis scheme for live automated cell imaging (LACI) using the open source program ImageJ to address these issues. Adult human muscle cells (hMCs) isolated by differential adherence rates were obtained from Cook Myosite (Pittsburgh, PA, USA). hMC populations (slow, moderate, and fast adhering), seeded in a multi-well plate, were imaged using a LACI system. Image sequences were imported to ImageJ for user interactive analysis of 17 parameters to describe cell morphology and behavior during in vitro culture. Analysis of LACI images showed significant differences in morphology between differentially adherent hMC populations for mean cell area, centroid velocity, and circularity. Mean cell area for fast adhering cells (1473±244um^2) was significantly smaller ($P<0.01$) than for slow adhering cells (2663±686um^2). In addition, both fast and slow adhering populations went through significant ($P<0.01$) increases in area (2555±721um^2 and 3659±832um^2 respectively) after the course of 5 passages. Analysis of centroid velocity showed slow adhering cells (0.987 um/min) exhibit a significantly greater in vitro centroid velocity than fast adhering cells (0.677 um/min). After the course of 5 passages, only the moderately adhering cells went through a significant increase ($P<0.01$) in centroid velocity (0.613 um/min to 1.094 um/min). In addition, the circularity of the fast adhering cells ($C=0.602$) was significantly different ($P<0.01$) than that of the slow adhering cells ($C=0.403$). The developed morphological measurement scheme has been shown effective to resolve populations of therapeutic hMCs, previously only resolved by way of adherence rates. Further expansion of this scheme may provide novel insight into the heterogeneous dynamics of the three populations and allow for more efficient isolation.

623/B585

Stable Expression of a Neuronal Dopaminergic Phenotype in Human Cell Lines Derived from Subcultures of Amniotic Fluid.

S. Pfeiffer, D. A. McLaughlin; Neuroscience, Conway Institute, University College Dublin, Dublin, Ireland

The foetal-maternal unit has long been considered a source of self renewing cells. Recently, we completed a long term program to establish and characterise cell lines derived from subcultures of human amniotic fluid cells from separate donors, obtained as a routine by-product of amniocentesis. Using a combination of techniques such as Flow Cytometry, Immunocytochemistry, RT-PCR and Western Blot analysis we have shown that these cells lack HLAII antigenicity and have consistent homogeneity in their molecular profile both among cell lines and within the same cell line over cell passing, the latest passage being 35. More specifically, our studies revealed weak expression of markers Nestin and Octamer-4 (OCT4) associated with early neuronal induction, yet strong expression of markers Microtubule-associated protein 2 (MAP2) and TAU for committed neurons, including Tubulin-BIII, with highly elongated, polarised processes similar to primary neuronal cultures. More recent studies have revealed strong expression of 5HT in early characterisation of these cell lines via immunocytochemistry, which is to be further examined by western blot analysis. Further evaluation for factors involved in dopaminergic neuronal development found selective expression of multiple factors concerned with inducing and maintaining dopaminergic cell fate and survival, such as Engrailed 1 (En1), Pentaxin-related protein PTX3, Glial-cell derived neurotrophic growth factor (GDNF) receptor c-RET and particularly Nuclear receptor related 1 (NURR1), and finally, genes regulating dopamine synthesis/secretion and neurotransmitter cycling such as L-aromatic amino acid decarboxylase (AADC), Vesicular Monoamine Transporter 2 (VMAT2), Syntaxin1A and SNAP25 (synaptosome-associated protein 25kDa). In conclusion, these data strongly support the idea that cells derived from human amniotic fluid possess a phenotype similar to committed dopaminergic neuronal progenitors and may be differentiated into a neuronal phenotype suitable for cell replacement therapies.

624/B586

Csx-1 and α-Myosin Heavy Chain Reporter Lines Identify Human Embryonic Stem Cell-derived Myocardial Precursors.

F. W. King[1], S. Y. Wong[1], C. Ritner[1], C. Ding[1,2], J. E. Olgin[1,2], H. S. Bernstein[1,3,4]; [1]Cardiovascular Research Institute, University of California, San Francisco, CA, [2]Medicine, University of California, San Francisco, CA, [3]Pediatrics, University of California, San Francisco, CA, [4]Institute for Regeneration Medicine, University of California, San Francisco, CA

Cell therapy with cardiomyocytes (CMs) derived from human embryonic stem cells (hESCs) have shown promise in animal studies of myocardial injury. While these cells survive transplantation, and appear to integrate electrically, it is unclear whether they truly augment myocardial tissue. Developmentally, such mature CMs may be beyond the ability to fully incorporate into existing muscle. Therefore, the identification and selection of myocardial precursors that are committed to the CM lineage, but retain the plasticity that would allow for complete engraftment remains an important goal. To address this, we constructed a lentiviral reporter encoding enhanced GFP under control of the mouse α-myosin heavy chain (αMHC) promoter, and used this to generate a stable hESC line by antibiotic selection. The engineered cell line grew normally on embryonic feeder cells and differentiated into all three embryonic germ layers, as assessed by immunostaining and quantitative PCR. However, cells expressing GFP/αMHC differentiated exclusively into CMs, as determined by spontaneous contractile activity, expression of myosin light chain 2 and cardiac troponin T in GFP-sorted cells, and optical mapping studies that demonstrated embryonic ventricular action potentials. Interestingly, cells expressing GFP/αMHC were observed at the onset of hESC differentiation, commensurate with the expression of the early cardiac transcription factors Nkx-2.5 and GATA4, and well before the expression of cardiac troponin T. These cells ultimately differentiated into CMs as described. In addition to the αMHC-GFP line, we generated a second reporter hESC line under control of the human cardiac homeobox Csx-1/Nkx-2.5 promoter. As expected, GFP positive cells were detected early during hESC differentiation. While characterization of this line is currently underway, our studies suggest that these reporter hESC lines will allow us to identify human myocardial precursors early during differentiation, before the expression of other sarcomeric proteins, and that they will provide powerful tools with which to study human cardiogenesis and select human myocardial precursors for cell transplant experiments.

625/B587

Cytokeratin 8 as a Novel Biomarker of Intervertebral Disc Cells.

A. Gilson, J. Urban; Physiology, Anatomy and Genetics, University of Oxford, Oxford, United Kingdom

Purpose: There is a growing interest in developing methods for repairing degenerated intervertebral discs by tissue engineering means; disc degeneration is highly associated with low back pain and is thus a major cause of disability in western industrialised societies. As there is no primary source of disc cells available, it has been suggested that autologous mesenchymal stem cells (MSCs) could be used for regeneration of the disc. However, poor knowledge of disc cell biology and the fact that MSCs can also differentiate into cells of other load-bearing cartilages such as articular cartilage chondrocytes, compromise this therapeutic development. Therefore, it is crucial to identify markers that could define a clear-cut phenotypic signature for disc cells. In this study, we have used a comparative proteomic approach in order to find specific markers for cells of the nucleus pulposus (NP), the central region of the intervertebral disc. Methods: Using 2-D fluorescence Difference Gel Electrophoresis technology, we compared protein contents from cells of the bovine nucleus pulposus (phenotypically similar to human disc cells) with bovine articular chondrocytes (AC). Results: A total of 14 differentially expressed proteins between the two cell types have been identified by mass spectrometry analysis. 12 proteins are specific to NP cells and 2 are specific to AC. Immunofluorescence analysis confirmed that one of the candidates, cytokeratin 8, is only expressed by NP cells although only a fraction of the cell population is positively stained. Conclusions: This study provides new molecular markers for use as a tool for monitoring differentiation of stem cells into disc cells. These markers will also be helpful in further understanding disc cell metabolism and therefore useful for any future disc tissue engineering.

626/B588

Expression of CD326 and CD14 Discriminate Distinct Populations of Hepatic Precursors in the Human Fetal Liver.

M. E. Fomin[1], L. Tai[1], M. O. Muench[1,2]; [1]Blood Systems Research Institute, San Francisco, CA, [2]Laboratory Medicine, University of California San Francisco, San Francisco, CA

The molecular profile of hepatic stem cells (HSC) and progenitors remains an incomplete, complicating their isolation and study. The epithelial cell adhesion molecule CD326 has emerged as a marker of hepatic precursors. In this study we sought to determine the breadth of expression of CD326 in the human fetal liver. Both CD45+ hematopoietic and CD45- non-hematopoietic cells expressed CD326. Diverse cell types expressing CD326 were revealed among CD45- cells by co-staining for CD14. A cell population expressing low levels of CD326 and no CD14 is the most promising candidate for HSC. These CD326loCD14- didn't express genes associated with functional hepatocytes but did express a set of cell surface markers related to hepatic progenitors such as CD117, CD34, CD44, CD26 and CD49f. In culture, under hepatic conditions, they expressed c-met, hepatic nuclear factor 3α (HNF3α), cytokeratins (CK) 19 and 7 and had the hepatocyte morphology. In different culture conditions these cells also gave rise to non-hepatic cells. Another population expressing the highest levels of CD326 and low levels of CD14 (CD326++CD14lo) comprised the bulk of fetal hepatocytes. CD326++CD14lo cells expressed hepatocyte markers including albumin, α-fetoprotein, c-met, HNF3α, prospero-related homeobox 1, cytochrome P450 3A7, α-antitrypsin and transferrin. Proteins expressed included CD24, CD26, CD29, CD34, CD49f, CD243, CD324, CK-7/8 and -19. CD326++CD14lo cells were not homogenous; they represented parenchymal cells, cholangiocytes and cells around ductal plates and large vessels. In culture, CD326++CD14lo cells yielded cells expressing albumin, CK19 or both markers. High levels of CD14 expression defined a third cell population. The CD326+ subset of CD14++ cells expressed a low level of hepatocyte and cholangiocyte makers and could generate cells expressing albumin and CK19 after culture, but most CD14++ cells represent another cell lineage, sinusoidal endothelial cells. In conclusion, CD326 is expressed by many cell types in the developing liver with a gradient of low to high expression marking hepatocyte differentiation. Co-expression of CD14 can be used to help identify functionally distinct subsets of fetal hepatocytes and their precursors.

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Differentiation of Nonhuman Primate Embryonic Stem Cells Along Neural Lineages.

X. Kuai[1], C. Gagliardi[1,3], M. Flaat[1,4], B. Bunnell[1,2,3]; [1]Division of Gene Therapy, Tulane National Primate Research Center, Covington, LA,

[2]Center for Gene Therapy, Tulane University Health Sciences Center, Tulane University, New Orleans, LA, [3]Department of Pharmacology, Tulane University Health Sciences Center, Tulane University, New Orleans, LA, [4]Department of Biochemistry, Tulane National Primate Research Center, Covington, LA

The differentiation of embryonic stem cells (ESCs) into neurons and glial cells represents a promising cell-based therapy for neurodegenerative diseases. Because the rhesus macaque is physiologically and phylogenetically similar to humans, it is a clinically relevant animal model for ESC research. In this study, the pluripotency and neural differentiation potential of a rhesus monkey ESC line (ORMES6) was investigated. ORMES6 was derived from an in vitro produced blastocyst, which is the same way human ESCs have been derived. ORMES6 stably expressed the embryonic transcription factors POU5F1 (Oct4), Sox2 and NANOG. Stage-specific embryonic antigen 4 (SSEA 4) and the glycoproteins TRA-1-60 and TRA-1-81 were also expressed. The embryoid bodies (EBs) formed from ORMES6 ESCs spontaneously gave rise to cells of three germ layers. After exposure to basic fibroblast growth factor (bFGF) for 14 to 16 days, columnar rosette cells formed in the EB outgrowths. Sox2, microtubule associated protein (MAP2),beta-tublinIII and glial fibrillary acidic protein (GFAP) genes and Nestin, FoxD3, Pax-6 and beta-tublinIII antigens were expressed in the rosette cells. Oct4 and NANOG expression were remarkably down regulated in these cells. After removal of bFGF from the medium, the rosette cells differentiated along neural lineages. The differentiated cells expressed MAP2, beta-tublinIII, Neuro D, and GFAP genes. Most differentiated cells expressed early neuron-specific antigen beta-tublinIII (73%±4.7%) and some expressed intermediate neuron antigen MAP2 (18%±7.2%). However, some differentiated cells expressed the glial cell antigens A2B5 (7.17%%±1.2%), GFAP (4.93%±1.9%), S100 (7%±3.5%) and O4 (0.27%±0.2%). The rosette cells were transplanted into the striatum of immune deficient NIHIII mice. The cells could be persisted for approximately 2 weeks and expressed Ki67, NeuN, MAP2 and GFAP. These results demonstrate that the rhesus monkey ESC line ORMES6 retains the pluripotent characteristics of ESCs and can be efficiently induced to differentiate along neural lineages.

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Self-Renewal and Expansion of Single Transplanted Muscle Stem Cells.

A. Sacco, R. Doyonnas, P. Kraft, H. M. Blau; Microbiology and Immunology, Stanford University, Stanford, CA

Adult muscle stem cells (MuSCs) play a major role in postnatal skeletal muscle growth and regeneration. Yet, much remains to be learned about the cell biological properties of MuSCs. These stem cells comprise a subset of satellite cells, that reside as quiescent cells underneath the basal lamina that surrounds muscle fibers and respond to damage by giving rise to transient amplifying cells (progenitors) and myoblasts that fuse with myofibers. In contrast to cultured myoblasts, when freshly isolated by flow cytometry and immediately transplanted into muscle tissues, MuSCs exhibit remarkable regenerative capacity. However, since MuSCs are known to be heterogeneous, a clonal analysis is required to demonstrate stem cell function. We have shown that when a single luciferase-expressing MuSC is transplanted into the muscle of mice, it is capable of extensive proliferation, and Pax7+luciferase+ cells can be readily reisolated, providing definitive evidence of self-renewal. In addition, we show using in vivo bioluminescence imaging, that the dynamics of MuSC behavior can be followed in a manner not possible using traditional retrospective static histological analyses. By imaging luciferase activity, real time quantitative and kinetic analyses show that MuSCs proliferate and engraft rapidly following injection until homeostasis is reached. Upon injury, transplanted mononucleated cells rapidly generate massive waves of cell proliferation. Together, these results show the dynamics of MuSC behavior and provide novel evidence at a clonal level that self-renewal is an autonomous property of a single adult muscle stem cell.

629/B591

Lentivirus Mediated Wnt11 Gene Transfer Enhances Cardiogenic Differention of Skeletal Muscle-derived Stem Cells.

G. Xiang, B. Wang, B. Gharaibeh, T. Cassino, L. Drowley, M. Okada, K. Tobita, J. Huard; Stem Cell Research Center, Children's Hospital of Pittsburgh and Department of Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA

Wnt signaling plays a crucial role in regulating cell proliferation and differentiation.Wnt11 is required for cardiogenesis and capable of inducing cardiomyogenesis in unfractionated bone marrow mononuclear cells. Skeletal muscle-derived stem cells (MDSCs) possess capability of differentiating into multilineages and potential in therapeutic application for myocardial infarction, however, whether Wnt11 can promote differentiation of MDSCs into cardiomyocytes remains uncertain. We here study role of Wnt11 in cardiogenic differentaion of MDSCs in vitro. Mouse skeletal MDSCs are obtained via a modified pre-plating technique and cultured in DMEM medium supplemented with 10% FBS, 10% horse serum and 0.5% chicken embryo extract. Lentivirus particles are produced by performing transient co-transfection involving a three-plasmid expression system in HEK293T cells and concentrated through ultra-centrifugation. After MDSCs were transduced with lentiviruses in the presence of 8 ug/ml polybrene, cardiac differentiation was evaluated by the expression of cardiac specific markers(transcription factors NKx2.5 and GATA4 , and cardiac electrophysiological coupling protein Connexin43) at the mRNA (semi-quantitative RT-PCR) and protein (immunocytochemistry and western blot) levels. Transduction of lentivirus bearing Wnt11 enables MDSCs to undergo morphological changes like becoming big and elongated, whereas control cells remain small and round shape. The Wnt11 transduction obviously increases mRNA expression of early cardiac markers NKx2.5 and GATA4, and gap junction protein Connexin43 (as detected as RT-PCR), and also protein expression of Nkx2.5 and Connexin43 (as shown as western blot and immunofluorescence), compared to the cells with treatment of empty lentiviruses. In addition, Wnt11 transduction leads to nuclear accumulation of NKx2.5 in some MDSCs, indicating increased transcriptional activity. Our results showed that Wnt11 enhances cardiomyogenic differentiation in MDSCs. This strategy might be utilized to induce and augment cardiac differentiation of MDSCs in infarcted myocardium after transplantation of transduced MDSCs.

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Preset TGF-β1 to Enlarge Population of Stem Cell-like Cells from Skeletal Muscle Cells of Mouse.

X. Mu[1,2], J. Ma[1,2], Y. Li[1,2], [1]Department of Orthopaedic Surgery, University of Pittsburgh Medical Center, Pittsburgh, PA, [2]Stem Cell Research Center at Children's Hospital, University of Pittsburgh Medical Center, Pittsburgh, PA

Much progress was recently made in the generation of induced pluripotent stem (iPS) cells with characteristics identical to those of embryonic stem (ES) cells from adult somatic cells. There are also somatic stem cells present in many differentiated tissues, such as skeletal muscle. Unlike pluripotent embryonic stem cells, somatic stem cells are generally limited to differentiate into different cell types of their

tissue of origin. However, somatic stem cell plasticity was suggested, for example, adult skeletal muscle stem cells can differentiate in to cartilage, bone, neuron, and skeletal muscle in different conditions. So that, as an alternative method of iPS cells, the induction of multipotent somatic stem cell by dedifferentiation of somatic adult cells can also be much beneficial for stem cell based therapies. We have already found that Ciliary neurotrophic factor (CNTF) could induce dedifferentiation of human myoblasts, which can be redifferentiated into adipocytes, glial, and neuronal cells. Here we investigated the dedifferentiation process in skeletal muscle cells promoted by TGF-β1. The Transforming Growth Factor (TGF)-β1 has been reported to induce dedifferentiation in axolotls (salamanders) and epithelial-mesenchymal transdifferentiation (EMT) in mammals. Our previous research also showed that TGF-β1 is responsible for the regulation of both the regeneration and fibrosis formation during the muscle healing process. Now we show that, after conditional TGF-β1 treatment, more proliferation-arrested C2C12 and primary myoblasts from mice were induced to re-enter the S-phase of cell cycle in vitro, and more stem-cell like cells e.g. positive for stem cell markers (Sca-1+/CD34+) were generated. In addition, the dedifferentiation marker protein-Muscle Segment Homeobox (Msx)-1 was exclusively expressed in the same cells with S-phase re-entering, which were further confirmed the process of dedifferentiation in these cells. This method may find a way to increase the population of multipotent stem cells for therapeutic applications.

631/B593

RPE Cells in Culture as "Stem Cells" to Produce Photoreceptors.

S. Wang, R. Yan; Ophthalmology, University of Alabama, Birmingham, Birminghma, AL

Photoreceptors are specialized sensory neurons in the retina, and their degeneration results in permanent blindness, because no effective therapies are available. One of the potential therapies is cell-replacement with health photoreceptors. This approach, however promising, faces a major roadblock - the lack of a supply of photoreceptors. In response to this critical need, we have been investigating a novel source of photoreceptors - the retinal pigment epithelium (RPE). RPE cells can be readily propagated in culture. In this study, RPE was isolated from chick eyes at embryonic day 6 (E6) and E15, when the RPE is approaching maturity. Dissociated RPE cells were cultured and then infected with a retrovirus (RCAS) expressing proneural gene ngn1. De novo production of photoreceptor-like neurons from the otherwise non-neural, RPE culture was examined at the molecular, cellular, and physiological levels. Here we report that strikingly high numbers of cells (as many as 90% of the cells in the culture) showed detectable photoreceptor traits under the induction of proneural gene ngn1. Reprogrammed cells expressed an array of photoreceptor genes and exhibited photoreceptor morphologies. They displayed hallmarks of photoreceptor cellular structures, such as well-formed inner segments. Excitingly, they responded to light by decreasing their cellular free calcium (Ca2+) levels. Further more, light-bleached, reprogrammed cells responded to 9-cis-retinal by increasing Ca2+ levels, reminiscent of visual cycle recovery. These molecular, morphological, and physiological traits support the exciting, provocative possibility of reprogramming RPE progeny cells to become photoreceptors for future cell-replacement studies.

632/B594

Human Mammary Progenitor Cell Fate Decisions Are Products of Interactions with Combinatorial Microenvironments.

M. LaBarge[1], C. Nelson[2], R. Villadsen[3], J. Ruth[1], M. Stampfer[1], O. W. Petersen[3], M. Bissell[1]; [1]Cell Biology and Imaging, Lawrence Berkeley National Lab, Berkeley, CA, [2]Chemical Engineering, Princeton University, Princeton, CA, [3]Medical Anatomy, Panum Institute, Copenhagen, Denmark

In adult tissues, multi-potent progenitor cells are some of the most primitive members of the developmental hierarchies that maintain homeostasis. That progenitors and their more mature progeny share identical genomes, suggests that fate decisions are directed by interactions with extrinsic soluble factors, ECM, and other cells, as well as physical properties of the ECM. To understand regulation of fate decisions, therefore, would require a means of understanding carefully choreographed combinatorial interactions. Here we used microenvironment protein microarrays to functionally identify combinations of cell-extrinsic mammary gland proteins and ECM molecules that imposed specific cell fates on bipotent human mammary progenitor cells. Micropatterned cell culture surfaces were fabricated to distinguish between the instructive effects of cell-cell versus cell-ECM interactions, as well as constellations of signaling molecules; and these were used in conjunction with physiologically relevant 3-dimensional human breast cultures. Both immortalized and primary human breast progenitors were analyzed. We report on the functional ability of those proteins of the mammary gland that maintain quiescence, maintain the progenitor state, and guide progenitor differentiation towards myoepithelial and luminal lineages.

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Propagation of Functional Muscle Stem Cells on Diverse Bioengineered Hydrogels.

P. Gilbert[1], K. H. Havenstrite[1], R. Doyonnas[1], A. Sacco[1], N. Nguyen[1], M. Lutolf[2], K. Koleckar[1], H. M. Blau[1]; [1]Microbiology and Immunology/Baxter Laboratories, Stanford University, Stanford, CA, [2]Ecole Polytechnique Federale de Lausanne, Lausanne, Switzerland

Adult stem cells comprise a reservoir of cells with a remarkable capacity to proliferate and repair tissue damage. Recently, procedures for isolating muscle stem cells (MuSCs), known as satellite cells, were developed and experiments testing their function upon transplantation into muscles revealed an extraordinary potential to contribute to muscle fibers and access and replenish the satellite cell compartment. However, MuSCs are a relatively rare cell type and their stem cell properties are rapidly lost once plated in culture limiting their clinical utility. We hypothesized that the long term culture of MuSCs on surfaces mimicking key features of the in vivo microenvironment would be able to increase their viability, promote division, and maintain their in vivo function. To this end, we employed a novel bioengineered hydrogel culture platform and assayed the in vitro and in vivo behavior of MuSCs cultured on hydrogels with different mechanical properties or tethered with different candidate niche proteins. We found that these variables greatly impact MuSC self-renewal potential, assayed upon transplantation in mice by a novel, noninvasive bioluminescent imaging technique as well as a classical histological approach. Our results are novel as they establish parameters for the long term culture and propagation of MuSCs in vitro that lead to the maintenance of MuSC function in vivo. Importantly, these studies should potentiate translation of stem cell biology to the clinic for the treatment of human muscle degenerative disorders.

634/B596

Blood-derived Dot Cells Regenerate Wounds by Self-Fusion before Undergoing Transdifferentiation.

W. Kong, S. Li, H. Lorenz; Surgery, Stanford University, Stanford, CA

Bone marrow-derived stem cells regenerate tissue by either transdifferentiation or fusion-dependent differentiation. We have identified a group of small bone marrow- and blood-derived "Dot cells". In vitro cell culture and immunofluorescent studies indicate that Dot cells express specific stem cell markers; they have a unique pattern of expansion by releasing grouped cells from large colonies or large spheroids. When co-present with differentiated cells, Dot cells aggregate and fuse together. The fused aggregates then undergo transdifferentiation to the differentiated cell type. In vivo studies demonstrate that transplantation of cultured male Dot cells through tail-vein injection into female diabetic mice significantly accelerates skin wound healing. Dot cells traffic to wounds and form spheroids, which release fused Dot cells expressing multiple y-chromosomes. Groups of fused cells then further differentiate into dermal cells. Almost every cell in the repair area expresses multiple y-chromosomes. These data indicate that Dot cells fuse together and then undergo transdifferentiation. Dot cells are the key fusogenic cells present in bone marrow and blood.

635/B597

Mechanical Control of Stem Cell Differentiation Using Micro-Engineered Matrix.

J. Fu, Y. Wang, C. Chen; Department of Bioengineering, University of Pennsylvania, Philadelphia, PA

In vivo, local tissue structure defines cellular environment, constraining how cells interact with surrounding extracellular matrix (ECM), neighboring cells, soluble growth factors, and physical forces. These "microenvironmental" cues in turn regulate the behavior of individual cells. In this work, we explore the molecular mechanisms by which local mechanical properties (e.g., rigidity) of ECM cooperates with soluble cues to regulate lineage commitment of human mesenchymal stem cells (hMSCs). By using semiconductor microfabrication techniques, we have established a standard suite of micropost array substrates that can definitively decouple matrix rigidity from other properties including adhesiveness. Using this approach, spatially defined regions of desired rigidity have been patterned with unprecedented precision. Our strategy involves replica-molding of arrays of PDMS posts from microfabricated silicon post arrays, and the rigidity of PDMS microposts can be precisely regulated by varying post height while keeping all other aspects of the substrate such as surface chemistry and ligand density unchanged. We have successfully used these substrates to investigate influences of matrix rigidity on cell adhesion, cytoskeleton assembly/contractility, cell spreading, proliferation and motility. We have further shown that matrix rigidity regulates commitment of hMSCs to either adipogenic or osteogenic fate: soft PDMS matrix facilitates adipogenic differentiation while stiff PDMS matrix proves osteogenic. Our results here suggest a strong functional interplay between stem cell differentiation, matrix rigidity and soluble factors, and will have great implications for understanding physical effects of the in vivo microenvironments in stem cell lineage specification and also for therapeutic applications of hMSCs.

Organogenesis (636-656)

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The SLIT2/ROBO1 (DUTT1) Signaling Axis Governs Branching Morphogenesis in the Mouse Mammary Gland.

H. Macias, A. Moran, R. Marlow, P. Strickland, L. Hinck; Molecular Cell and Developmental Biology, University of California Santa Cruz, Santa Cruz, CA

Our laboratory has been investigating the role of SLIT/ROBO signaling in epithelial morphogenesis using the mouse mammary gland as a model system. The mammary gland consists of an epithelial ductal structure surrounded by a stroma containing adipocytes, fibroblasts, immune and blood cells. SLITs are a family of secreted ligands that were originally discovered in the nervous system where they control both axon guidance and branching through their ROBO receptors. We have previously shown that SLIT2 and SLIT 3 are expressed in the mammary gland along with their ROBO receptors. We sought to determine the role, if any, played by the SLIT/ROBO1 signaling axis in regulating mammary branching morphogenesis. Analysis of whole glands harboring loss-of-function mutations in Slit2 and Slit3 or their Robo1 receptor revealed a similar hyper-branching phenotype. Since TGFβ plays a key role in inhibiting mammary gland branching, we evaluated its expression by Western analysis and observed decreased levels in lysates of Robo1-/-, compared to wildtype, glands. Next, we generated primary cultures and noted an upregulation of Robo1 in wildtype cells upon TGFβ addition. Primary cells were also cultured in matrigel to allow acini formation. As previously described, wildtype cells do not branch after 3 days in culture, whereas Robo1-/- cells precociously formed elaborate branched structures. Taken together, these studies indicate an interplay between SLIT and TGFβ signaling that regulates the generation of branches during mammary gland development.

637/B599

Molding the Murine Lung: Decoding the Molecular Basis of Lung Branching Morphogenesis Using Functional Genomics and Live Imaging.

C. Schnatwinkel, A. Minic, L. Niswander; Pediatrics, HHMI/Univ. of Colorado at Denver and Health Sciences Center, Aurora, CO

A major goal in understanding organ formation is determining the link between developmental signals and the molecular pathways responsible for guiding and controlling organogenesis. Although much is known about the molecules that initiate morphogenesis, including Wnts, Hedgehogs, BMPs and growth factors, there is a considerable gap in understanding the downstream targets of these regulators, the links between them, and the mechanisms by which these regulators generate three-dimensional tissues such as the branching pattern of the lung. In order to fill this gap we developed a systems approach to obtain a comprehensive understanding of the functional relationship between kinase/phosphatase-regulated molecular pathways and their roles in organizing the branching pattern of the mouse embryonic lung. To this end, we devised a combination of a loss of function screen with morphological and live imaging analysis in lung explant cultures. Our studies reveal various morphological phenotypes, suggesting distinct morphological, molecular and mechanistic phases during the process of branching morphogenesis. Based on our observations, we have divided a single bifurcation event into four morphological and molecularly

distinct stages. (1) The bud stage, where the size of the initial epithelial bud is increased. (2) The flattening stage, where the round bud becomes flattened. (3) The bifurcation stage: epithelial cells on the two sides of the bud begin to branch, whereby cells in the middle are constrained and remain in place. (4) The outgrowing stage in which the two distinct branches continue their directional migration and/or growth. Molecularly, this process is in part regulated by phosphorylation of the actin severing protein cofilin. Whereas cofilin remains in its active desphosphorylated state during the bud outgrowth, it becomes increasingly phosphorylated during bifurcation in the region between the branches to constrain the cells in place. Thus, the combination of functional genomics and live imaging provides a powerful strategy to further decipher the molecular networks and principles underlying branching morphogenesis and may help to decode the morphogenetic information provided by the known signalling molecules.

638/B600

Cardiac Jelly Facilitates Cellular Communication between Endocardium and Myocardium during Heart Looping.

B. Garita[1], M. W. Jenkins[2], A. M. Rollins[2], M. VanAuker[3], K. K. Linask[1]; [1]Pediatrics, Univ South Florida, St. Petersburg, FL, [2]Biomedical Engineering, Case Western Reserve University, Cleveland, OH, [3]Chemical Engineering, Univ. South Florida, Tampa, FL

Throughout looping stages of cardiac development, the myocardium (MYO) and endocardium (ENDO) are separated by a thick layer of cardiac jelly. During looping the heart undergoes structural changes driven by cell movements, cell division, cytoskeletal reorganizations and, we suggest, blood flow. Part of the structural changes during looping may result from pressure forces imposed by increasing blood flow on the endocardium endothelium. The cardiac jelly represents a mediating substrate allowing for cell communication and mobility critical in the communication between ENDO and MYO during diastole and systole. To analyze cardiac shape changes during the cardiac cycle, we use ultra high speed optical coherence tomography that allows capturing 235 frames/second in comparison to the 50 frames/second presently reported by others. This technology allows real time visualization of the movement and the interrelationships of the ENDO, cardiac jelly, and the MYO in the avian heart through one complete cardiac cycle. We determined the presence of endocardial folds that increase the circumferential endothelial surface at diastole. Using 3D reconstruction of looping hearts and segmentation of the three compartments, we also demonstrate a complexity in the ENDO and the lumen shape that has heretofore not been reported. Histological analysis of immunostaining for cytoplasmic nonmuscle myosin II, a molecule associated with cytoskeletal mechanosensing, revealed myocardial cell processes extending from the myocardial wall radially toward the ENDO. In regions along the myocardial basal lamina the cell processes interact with fibronectin. Also, we isolated cardiac jelly/myocardial fragments and observe in the myocardial fragments similar long cell processes extending away from the wall. In summary, we propose the existence of these long cell processes within the cardiac jelly suggests a role in their facilitating communication of forces relating to blood flow in the endocardial lumen to the myocardium, as the MYO wall changes in shape by contracting and elongating, and moves to form the four-chambered heart.

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Gata5 Cooperatively Regulates Cardiomyocyte Proliferation in Mice.

Y. Li[1], D. Zhou[2], M. Lu[2], E. Morrisey[2], P. J. Gruber[1,2]; [1]Children's Hospital of Philadelphia, Philadelphia, PA, [2]University of Pennsylvania School of Medicine, Philadelphia, PA

The Gata family of transcription factors plays critical roles in vertebrate development. Gata5 is expressed during early embryogenesis in the primitive endoderm as well as the pre-cardiac mesoderm. Within the heart, it is expressed in endocardial cells until mid gestation, after which it is extinguished. In other organs, it persists in the lung, gut epithelium, and urogenital ridge. The faust/gata5 zebrafish mutant, characterized by the defects in endocardial and myocardial differentiation and migration, suggested an important role for Gata5 in the endoderm and heart. However, deletion of exon 1 from the mouse Gata5 gene has a relatively minor effect, with only females harboring genitourinary abnormalities. Our objective was to understand the cooperative role Gata5 might play in cardiogenesis. We generated a new Gata5 allele lacking of exons 2 and 3 that encodes the zinc finger region hypothesizing that this Gata5 null allele would yield additional defects. Homozygous Gata5 mutants were viable with females displaying genitourinary abnormalities, similar to the exon 1 deletion mutant. Homozygous females were fertile, but died of dystocia, whereas homozygous male mutants were grossly normal. To determine whether Gata4 or Gata6 might compensate for Gata5 function in mutant mice, we created Gata4+/-,5-/- and Gata5-/-,6+/- double mutants. Gata4+/-,5-/- mice died mid-gestation while Gata5-/-,6+/- mice died perinatally. Characterization of multiple, mutant alleles revealed a complex group of non-overlapping intracardiac defects (Gata5 vs. Gata45 vs. Gata56) suggesting only partial Gata factor redundancy, not sufficiency. Myocyte proliferation was decreased in Gata5 null compound mutants compared to Gata4 or Gata6 heterozygous mutants suggesting that Gata5 plays a redundant role in the heart regulating proliferation. Additionally, since the Gata4+/-,5-/- and Gata5-/-,6+/- double mutants harbored distinct cardiac abnormalities, this suggests the possibility of specific rather than redundant roles for Gata4, 5, and 6 in cardiac development A deep understanding of Gata factor biochemistry in the heart will be required to dissect the pathways that mediate cardiac morphogenesis.

640/B602

Egfl7 Knockdown Causes Defects in Endothelial Cells during Zebrafish Vasculogenesis.

A. M. De Mazière[1], L. Parker[2], S. van Dijk[1], W. Ye[2], J. Klumperman[1]; [1]Cell Microscopy Center, Department of Cell Biology, UMC Utrecht and Institute for Biomembranes, Utrecht, Netherlands, [2]Tumor Biology and Angiogenesis Department, Genentech Inc., South San Francisco, CA

The endothelial-cell-specific, secreted protein EGFL7 plays a role in lumen formation during vasculogenesis. To characterize at the ultrastructural level the defects in tube formation caused by *Egfl7* KD, we performed a quantitative electron microscopy study of the developing axial vessels in zebrafish embryos. At 24 hours post fertilization (hpf), endothelial cells (ECs) of the dorsal aorta (DA) and posterior cardinal vein (PCV) correctly associated with hypochord and endoderm, respectively, but they failed to spread into the vascular area. This resulted in open sheets of ECs and vessels with reduced or split lumen. Besides normal tight junctions between adjacent ECs, ectopic cell junctions occurred across the obliterated vessel lumen, between the luminal EC surfaces, which were observed in none of the wild type embryos. These data suggest that EGFL7 provides ECs with a cue for their extension in the vascular area and for establishing cell polarity. Concomitant with the aberrant positions of ECs in the *Egfl7* KD embryos, the normal arrangement of red blood cell (RBC)

precursors in the vessel lumen and in a layer between DA and PCV was disturbed. We confirmed the erythroid nature of these cells by performing immunogold labelling for GFP on ultrathin cryosections of embryos of a *GATA1:eGFP* transgenic line. The presence of GFP correlated with the occurrence of hitherto not described striated membrane tubules in the peripheral cytoplasm of these cells. The tubules had an inner coating of the membrane, and they were occasionally clathrin-coated on one end and connected to the plasma membrane. Analogy with tubules in rat bone marrow erythroblasts suggested that the tubules contain transferrin receptor, involved in iron uptake. Due to their characteristic structure and abundance, the tubules may be used as ultrastructural markers for zebrafish RBC precursors.

641/B603

An In Situ Hybridization Screen Reveals New Transcriptional Regulators of Third Pharyngeal Pouch and Thymic Epithelial Cell Differentiation.

Q. Wei, B. Condie; Genetics, University of Georgia, Athens, GA

Thymic epithelial cells (TECs) are the major component of the non-lymphoid framework of the thymus and are essential for normal T cell development. In mice, TECs develop from a domain of the embryonic third pharyngeal pouch endoderm marked by Foxn1 expression. Although Foxn1 is necessary for proliferation and differentiation of TECs, it is not required for specification of TECs from the pouch endoderm. Also, some aspects of TEC differentiation are Foxn1-independent. This suggests that there are additional transcription factors expressed within the third pouch endoderm that are responsible for early specification and differentiation of TECs. To identify additional transcriptional regulators of third pouch and TEC differentiation, we performed an in situ hybridization screen on a set of candidate transcription factors identified through a systematic analysis of gene expression databases. We found that most of the genes selected for detailed characterization were expressed in the third pharyngeal pouch endoderm and many of them were specifically expressed in the thymus domain of the pouch. These transcription factors are highly likely to be involved in the earliest steps of TEC development. Our work also shows that data mining of gene expression databases is an efficient way to identify genes involved in the development of a specific tissue or organ.

642/B604

An Allele on Chromosome I Affects C. elegans Pharynx Muscle Cell Morphology.

A. Charron, E. Pahomov, P. Smith; Biology, Lake Forest College, Lake Forest, IL

The development of any organism is directed by sequential gene expression that provides a basis for differentiation and specialization of cells. Our lab is interested in the genes that control organogenesis, specifically, the development of the C. elegans pharynx. The microscopic nematode Caenorhabditis elegans is a model organism that provides many advantages for this study. Its complete cell lineage is known, it is transparent, it has a short life cycle and it is ideal for genetic experiments. Our lab previously performed a mutagenesis screen that resulted in over 200 defective pharynx phenotypes. We have selected two strains to investigate, M136 and M138, which are characterized by extreme deformation of the pharynx resulting from possible failure of pharyngeal muscle cells to undergo normal morphogenesis. We hypothesize that the phenotype may be a result of abnormal adhesion of cells and resulting in L1 larval lethality. We are addressing the lethality using multiple methods, immunocytochemistry to reveal the structure of the abnormal pharynx, fluorescent bead feeding assays to determine if the pharynx has function, and genetic mapping to reveal the identity of the gene involved. To determine the gene mutated in this pharynx phenotype, we used single nucleotide polymorphism mapping to elucidate the chromosomal location of the allele. Chromosomal mapping localizes the mutation to chromosome 1. A complementation analysis has shown M136 and M138 to be caused by the same mutation. Future research will entail complementation with deletion strains to determine the exact location of the mutation, followed by the use of transgenic rescue microinjections to confirm the identity of the gene.

643/B605

The Transcriptional Control of Secretion in the Drosophila Salivary Gland.

R. M. Fox, D. J. Andrew; Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD

Many specialized organs require high-level secretory activity to perform their normal functions; the genetic mechanisms through which a cell develops the capacity to secrete large quantities of protein is, however, poorly understood. In the *Drosophila* embryo, the salivary gland is the largest secretory organ, making it an excellent system to study the acquisition of high-level secretory function. Among several transcription factors expressed in the secretory portion of the salivary gland is CrebA, which we have shown to be required for the elevated expression of 34 known secretory pathway component genes (SPCGs). 33 of the 34 SPCGs contain putative CREB consensus sites within 2 kb of their translational start sites and electrophoretic mobility shift assays reveal that purified CrebA protein binds directly to the consensus sites *in vitro*. This interaction is specific, since mutating conserved nucleotides within these sites abolishes CrebA binding. Direct CrebA regulation of SPCG expression through these consensus sites is currently being tested *in vivo*. Given the requirement for *CrebA* to promote high-level SPCG expression, we ascertained the role of CrebA within the cell, using both electron microscopy and fluorescence imaging. Loss of *CrebA* results in dramatic and unexpected changes in organelle shape and localization. As predicted, the number and size of secretory vesicles are significantly reduced in the *CrebA* mutant salivary gland. Conversely, over-expression of *CrebA* expands the intracellular components of the secretory pathway, based on increased levels of staining with organelle-specific markers. Thus, through its regulation of both the integrity of intracellular organelles and the expression of secretory pathway components, CrebA is the key regulator of salivary gland secretory function.

644/B606

Essential Requirement for miRNA Processing in Vertebrate Pancreas Development.

B. Ghosh, S. Scott, S. Leach; Surgery, John Hopkins University School of Medicine, Baltimore, MD

Objective: The long-term goal of this study is to elucidate the role of microRNAs (miRNAs) in vertebrate pancreas development and disease. Recently a number of studies have implicated miRNAs as critical regulators of diverse biological processes including cell proliferation, apoptosis, lineage specification, embryonic development and cancer. However, the role that miRNAs might play in regulating pancreatic development has not been fully explored. Methods: To assess the role of miRNAs in mouse pancreas development we conditionally deleted

Dicer (a key enzyme required for miRNA processing) in mouse exocrine pancreas by crossing *Ela-Cre* ER^{T2} mice with floxed *Dicer* mice and induced deletion of *Dicer* by administering tamoxifen. We have also performed global deletion of *Dicer* by crossing *Rosa-Cre* ER^{T2} mice with floxed *Dicer* mice. We have identified and examined an exocrine pancreas specific microRNA miR-217 following microRNA microarray in mouse developing pancreas. Results: Pancreatic tissue in *Ela-Cre;* $Dicer^{loxp/loxp}$ mice displayed no gross defects following treatment with Tamoxifen as compared to oil treated control. Immunoflorescence analysis for exocrine marker Amylase reveals that lack of Dicer in the acini tissue led to the reduction of Amylase protein. However, global deletion of *Dicer* led to profound histological changes in exocrine pancreas in the tamoxifen treated mice as indicated by smaller and disorganized acini with loss of zymogen granules. Quantitative PCR analysis of miR-217 across developing mouse pancreas revealed maximum expression in adult while immunoflorescence following over expression of miR-217 in dorsal bud explants showed disorganized epithelial structure. Conclusion: miRNA processing is absolutely required for mouse pancreas development and the observed histological changes in the exocrine pancreas in the absence of Dicer may depict miRNA's direct role in pancreatic development and diseases. MiR-217 expression in the adult pancreas at a significant high level may suggest its role in the maintenance of acinar tissue rather than differentiation.

645/B607

Alpha 2-Macroglobulin Regulation of Axial and Gut Morphogenesis in Xenopus laevis.

L. Pineda-Salgado, D. Kessler; Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA

Alpha 2-macroglobulin (α2M) is a major serum protein that inhibits protease activity and binds growth factors. In Xenopus laevis, two α2M genes, Endodermin (Edd) and Panza, have been isolated. Edd is expressed in endoderm and dorsal mesoderm cells and with the onset of gut coiling expression is restricted to the liver. In contrast, Panza is expressed in the dorsal domain of the gut endoderm. During gut coiling Panza expression is initiated in the liver and is maintained in the liver as Panza expression is lost from other gut regions. The overlapping expression patterns of Edd and Panza in gut endoderm suggests that α2M function may be important for differentiation and morphogenesis of the gut. To examine the role of Edd and Panza in gut development, loss-of-function experiments using morpholino oligos (MO) were performed to knockdown Edd and Panza individually and together. Panza knockdown resulted in a ventral bending of the posterior axis, lack of gut coiling, kinking of the notochord and somites defects. Edd knockdown resulted in a shortened body axis, a failure of tail outgrowth, lack of gut coiling, notochord shortening and somites defects. Knockdown of Edd and Panza resulted in a dramatically shortened body axis, an absence of tail structures, head and eyes defects, lack of gut coiling, notochord degeneration and disorganized somites. Mesodermal and endodermal patterning was examined from the gastrula stage through the coiled gut stage in single and double knockdown embryos. Formation and patterning of the mesoderm and endoderm was unaffected by Edd or Panza knockdown. These results suggest that α2M function is essential for axial and gut morphogenesis by influencing the shape and movement of cells and tissues, rather than by perturbing developmental patterning of mesodermal and endodermal tissues.

646/B608

Developmental Mechanism of Serosal Mesothelium Formation.

N. Winters, E. Shelton, D. Bader; Cell and Developmental Biology, Vanderbilt University, Nashville, TN

The objective of this study was to establish the developmental stage and mechanism by which the definitive serosal mesothelium forms over the gut tube in chick embryos. In situ hybridization (ISH) and immunofluorescence were used to assess the phenotype of the surface layer of the gut tube as development progresses. Vital dye labeling and tracing of surface cells of HH21-31 chick embryo gut tubes were performed by injection of vital dye into the peritoneal cavity of embryos in ovo or by culturing dissected and labeled gut tubes in vitro. Labeled embryos/gut tubes were analyzed by immunofluorescence. Expression of Wilms tumor protein (Wt1) is commonly used as a marker of mesothelium, the outermost simple squamous epithelium of the gut. ISH analysis of the chick midgut shows that Wt1 is expressed in the mesentery (HH29) prior to expression over the gut tube (HH31) suggesting a relatively late appearance of the mesothelium and the possibility of a migratory population. In contrast, as early as HH21—long before mesothelial markers are expressed, two basement membranes exist dividing the gut tube into three regions: the inner endodermal epithelium, a middle mesenchymal layer, and an outer epithelial layer. The outermost epithelial layer initially is stratified but as development progresses appears to go through the transitions of pseudostratified, to columnar, to cuboidal, and finally to squamous. The persistence of a basement membrane throughout the change in morphology raises the question of the mechanism of mesothelial formation in the gut tube. Vital dye labeling of the gut tube can be used to differentiate between three of the major possibilities: 1) the outermost stratified layer is the mesothelium precursor that becomes squamous secondary to the rapid growth of the gut tube, 2) a resident mesothelial precursor within the outer epithelium is left behind as the remaining cells undergo epithelial-mesenchymal transition, or 3) an exogenous source of mesothelium migrates over the gut tube later in development. In conclusion, the mechanism of mesothelium formation in the gut is unresolved and direct labeling techniques are necessary to establish definitively the origin of this important cell type in development.

647/B609

Flow-dependent Epithelial Cell Migration Drives Kidney Nephron Morphogenesis.

A. Vasilyev[1], K. Poon[2], I. Kondrychyn[2], V. Korzh[2], I. A. Drummond[1]; [1]Medicine and Genetics, MGH/Harvard Medical School, Charlestown, MA, [2]Institute of Cell and Molecular Biology, Proteos, Singapore

Tissue organization in epithelial organs is achieved during development by the combined processes of cell differentiation and morphogenetic cell movements. In the kidney, the nephron is the functional organ unit. Each nephron is an epithelial tubule that is sub-divided into discrete segments with specific transport functions. Little is known about how nephron segments are defined or how segments acquire their distinctive morphology and cell shape. Using live, in vivo cell imaging of the forming zebrafish pronephric nephron, we found that migration of fully differentiated epithelial cells accounts for both the final position of nephron segment boundaries and the characteristic convolution of the proximal tubule. Pronephric cells maintain adherens junctions and polarized apical brush border membranes while they migrate collectively. Individual tubule cells exhibit basal membrane protrusions in the direction of movement and appear to establish transient, phopho-FAK-positive adhesions to the basement membrane. Cell migration continued in the presence of hydroxyurea, indicating cell

division does not drive migration. Lengthening of the nephron was however accompanied by an increase in tubule cell number specifically in the most distal, ret1-positive nephron segment. Initiation of cell migration coincided with the onset of fluid flow in the pronephros. Complete blockade of pronephric fluid flow prevented cell migration and proximal nephron convolution. Selective blockade of proximal, filtration-driven fluid flow shifted the position of tubule convolution distally and revealed a role for cilia-driven fluid flow in persistent migration of distal nephron cells. We conclude that nephron morphogenesis is driven by fluid flow-dependent, collective epithelial cell migration within the confines of the tubule basement membrane. Our results establish intimate links between nephron function, fluid flow, and morphogenesis.

648/B610

The BMP Receptor, ALK3, Is Required for Urogenital Development.

V. E. Di Giovanni[1,2,3], A. Alday[1], Y. Mishina[4], N. Rosenblum[1,2,3]; [1]Program in Developmental and Stem Cell Biology, Hospital for Sick Children, Toronto, ON, Canada, [2]Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada, [3]Division of Nephrology, Hospital for Sick Children, Toronto, ON, Canada, [4]Reproduction and Developmental Toxicology, NIH, Bethesda, MD

The mammalian kidney and reproductive systems both derive from a common embryological origin, the intermediate mesoderm (IM). Abnormal IM development can result in congenital anomalies of the urogenital system, yet the molecular mechanisms that govern IM development are incompletely defined. The spatial and temporal expression of the proteins BMP-2 and 4 and their ALK-3 receptor in urogenital tissues, suggests a function for BMP-ALK3 signaling. Our objective is to determine the role of ALK3 in urogenital development. We created mice with a deficiency of the BMP receptor, ALK3, restricted to the renal metanephric mesenchyme (MM) cell lineage and mesonephric (MN) tubule derived structures of the testis. Mice containing a conditional *Alk3* allele, *(Alk3flox/flox)*, were crossed to *Alk3+/-* mice expressing CRE directed by the *Rarb2* promoter, targeting excision to the renal MM and the MN cells. To study renal morphogenesis, embryonic kidneys were analyzed by histology and immunolabeling of molecular markers for nephrogenic intermediates. 115 mutants *(Rarb2Cre;Alk3 -/flox)* were identified at ages spanning E12.5 to 1 year among 496 mice. This ratio is consistent with Mendelian segregation and demonstrates viability of nulls. Histology revealed small renal size in *Alk3* null kidneys, and fewer nephrogenic intermediates and glomeruli, consistent with renal hypoplasia. These results were supported by a 50% decrease in NCAM+ structures at E12.5 (intermediate nephrogenesis marker), and 75% fewer WT-1+ structures (podocyte marker) at E16.5. The decrease in nephrogenesis was not associated with increased apoptosis or reduced proliferation in E12.5 *Alk3* null kidneys. *Alk3* null kidneys showed decreased *Osr1* expression, an IM gene required for renal lineage commitment. Staining for phospho-p38, a kinase downstream of ALK3, exhibited reduced expression in early *Alk3* null kidneys. Testes from *Alk3* nulls had a paucity of MN-derived seminiferous tubules and reduced organ size. Marker analysis revealed fewer testosterone producing MN-derived Leydig cells. In contrast to the kidney, *Alk3* null testis tissue showed less phospho-Smad1/5/8 staining. We conclude that ALK3 is required during nephrogenesis of the mammalian kidney and in testes.

649/B611

Occurrence and Distribution of Osteoclasts during the Morphogenesis of Avian Tarsometatarsal Bone.

Y. Namba, Y. Yamazaki, M. Yuguchi, K. Isokawa; Anatomy, Nihon University School of Dentistry, Tokyo, Japan

Avian tarsometatarsal (TMT) bone equivalent to distal tarsus plus metatarsal bones in human is formed by fusion and elongation of several primordia. We reported temporal pattern of TMT bone morphogenesis and the coordinated changes in the distribution of osteoblasts previously in this meeting. In the present study, we show the occurrence and distribution of osteoclasts in the TMT bone development from embryonic day 6 (ED6) to ED20 by utilizing tartrate resistant acid phosphatase (TRAP) staining on undecalified frozen sections prepared according to Kawamoto's method. The results showed that osteoclasts were observed in the mid-diaphysis at ED9-10, while the calcification of TMT bone collar (BC) was initiated at ED8, indicating that osteoclasts emerge about two days after the onset of BC calcification. Osteoclasts were observed in the surface of inner and outer cylinders of BC and their connecting-struts. Occurrence of osteoclasts appears to be coordinated to the initial invasion of vasculature into cartilageous core in the mid-diaphysis. The increase of the number of trabeculae and channels of BC results in the radial growth and eventual fusion of initially-independent TMT primordia. Osteoclasts were absent in the surfaces for fusion, but endosteal surface and eroding cartilage were populated by TRAP-positive cells. These findings suggest that the lateral fusion of primordia and radial growth of TMT bone can be attained by well-regulated coordination between 1) degradation of cartilage and endosteal side of bone by osteoclasts and 2) apposition of bone by osteoblasts aligned in the perimeter of developing TMT.

650/B612

The Role of GPI-anchored Proteins in Chondrogenesis and Cell Polarity.

M. Ahrens, Y. Li, A. T. Dudley; BMBCB, Northwestern University, Evanston, IL

Proteins that are localized to the cell surface via glycosylphosphatidylinositol (GPI) linkages have been proposed to regulate cell signaling and cell adhesion events involved in tissue patterning. Here we demonstrate that during skeletal development GPI-anchored proteins play limited, focal roles in chondrogenesis. Conditional deletion of Piga, an essential enzyme in the GPI-biosynthetic pathway, in the lateral plate mesoderm results in limbs that display chondrodysplasia. Analysis of mutant and mosaic Piga cartilage revealed two independent cell autonomous defects. First, loss of Piga function interferes with signal reception by cells and results in delayed chondrocyte maturation. Second, the proliferative chondrocytes, while present, fail to flatten and arrange into columns of clones as in wildtype tissue. We present evidence that the abnormal organization of mutant proliferative chondrocytes results from errors in cell intercalation following cell division. Consistent with the known relationship between cell polarity and cell intercalation, we additionally show that GPI-anchored proteins regulate cell polarity in other tissues. Collectively, our data suggest that the distinct morphological features of the proliferative chondrocytes result from regulated cell polarity that is controlled independent of chondrocyte maturation.

651/B613

Zebrafish Glypican 4 (Knypek) and Wnt5b (Pipe tail) Control Chondrocyte Behavior Essential for Craniofacial Cartilage Morphogenesis.

J. Topczewski; Pediatrics/CMRC, Northwestern University Feinberg School of Medicine, Chicago, IL

The Wnt/PCP pathway has been shown to control cell polarity and cell behavior in both invertebrates and vertebrates. Glypicans, a class of membrane-linked heparan sulfate proteoglycans, interact with secreted signaling molecules, including Wnt/PCP ligands, and modulate their signaling potential. We are studying the role of Glypican4 and Wnt5b in craniofacial skeleton morphogenesis. The early zebrafish skull is composed predominantly from very simple cartilage elements. We have found that the *glypican4* (*knypek*) and *wnt5b* (*pipe tail*) mutations result in dramatically misshapen head cartilage. We identified a loss of chondrocyte polarity in *knypek* and *wnt5b* mutants as the cellular basis of the observed defect. This results in a failure of cell-cell intercalation and consequent defects in elongation of cartilage elements. Interestingly, cartilage elements that are formed by intercalation independent mechanisms are relatively normal, indicating a requirement for Wnt5b/Knypek function in a specific type of chondrocyte behavior. In both mutants chondrocyte precursors fate determination, cell migration, and cell proliferation were not altered. These cell behavior problems resemble well-characterized defects observed in *knypek* and *pipe tail* mutants during the convergence and extension movements of gastrulation. Our study of adult *knypek* homozygotes uncover postembryonic role of Glypican4 in cartilage organization and bone formation. We have determined that the growth plate of adult *knypek* mutants lack a chondrocyte stacking zone. Deficiency in normal Glypican4 function also result in malformation of adult craniofacial bones. We identified both subtle alterations, including shortening of the rostral-most skull, and much more dramatic abnormalities including loss of the symplectic, one of the zebrafish facial bones. We propose here that those adult bone defects are, at least in part, a consequence of the larvae cartilage morphogenesis problems. Our results for the first time implicate Glypican4 and Wnt5b in the control of chondrocyte polarized behavior and suggest novel function of Wnt/PCP signaling in vertebrate bone formation.

652/B614

Hox-independent Roles of Pbx Homeoproteins in Midface Morphogenesis.

E. Ferretti, R. Zewdu, L. Selleri; Cell & Developmental Bio, Weill Cornell Medical College, New York, NY

One third of all human congenital malformations affect craniofacial development and represent a serious concern for society. Within the craniofacial birth defects, Cleft Lip with or without Cleft Palate (CL/P) has an occurrence of 1 in 500 to 2,500 live births worldwide, and represents the most common craniofacial birth defect in humans. Mutant mice deficient for multiple Pbx genes are an unique model to study CL/P disorders. Indeed, the Pbx compound mutant embryos exhibit abnormalities in the formation of the upper lip and secondary palate, resulting in bilateral cleft lip and palate. The data that we have collected so far demonstrate that mammalian midface morphogenesis requires multiple Pbx family members (Pbx1, Pbx2 and Pbx3), and that Pbx2 and Pbx3 genetically interact with Pbx1. As we did not observe craniofacial abnormalities in Pbx compound mutant embryos, unless both alleles of Pbx1 were inactivated, we concluded that Pbx1 plays a major role in midface development. Concomitantly, Pbx2 and Pbx3 exert collaborative roles with Pbx1 in craniofacial domains wherein they are co-expressed. Additionally, Pbx1, Pbx2 and Pbx3 proteins co-localize in the developing frontonasal process, where they are present at high levels in facial and nasal ectoderm and at lower levels in the mesenchyme. In addition, we demonstrated that restricted, tissue-specific impairment of Pbx function in midfacial ectoderm is sufficient to cause abnormal lip and palate fusion. Indeed, specific inactivation of Pbx1 in the midfacial ectoderm (obtained by crossing Pbx1 conditional mice with the Bf1 Cre deleter strain) showed that Pbx1 flox/flox ;Pbx2+/- ;Bf1Cre+ mutant embryos exhibit bilateral CL/P. Interestingly, specific inactivation of Pbx1 in the neural crest-derived midfacial mesenchyme (generated by crossing Pbx1 conditional mice with the Wnt1 Cre deleter strain) demonstrated that Pbx1 flox/flox ;Pbx2+/- ;Wnt1Cre+ mutant embryos do not reveal any evident midfacial phenotype. These results indicate that Pbx1 exerts a fundamental and preferential role in the midfacial ectoderm for proper morphogenesis. Last but not least, the Pbx compound mutant mice represent a unique model to study CL/P in mammals.

653/B615

Caspase-8 Regulates Intercellular Crosstalk during Tissue Regeneration.

C. Jamora, P. Lee, D. Lee; Biology, University of California, San Diego, La Jolla, CA

Wound healing in the skin is a highly orchestrated process that involves inflammation, cell proliferation, reorganization of the extracellular matrix, and tissue remodeling. This complex procedure is mediated by the reciprocal exchange of cytokines and growth factors between epithelial and mesenchymal cells and leukocytes. Many of the players in tissue repair and regeneration have been identified, but how they are coordinated to guide this multifactorial program remains an open question. We found that the serine protease caspase-8, which plays a role in transmitting external cues to activate the apoptotic pathway, has an unexpected role in mediating many features of the wound healing response. Caspase-8 is expressed near the surface of the skin and is downregulated upon wounding. Using a conditional knockout mouse model, we found that loss of epidermal caspase-8 leads to the proliferation of pools of cutaneous stem cells and promotes the survival of differentiated keratinocytes. This effect is initiated by interleukin-1α (IL-1α) signaling on multiple cell types that stimulates the release of other cytokines. Among the downstream effects of this intercellular communication are the potentiation of stem cell proliferation as well as the infiltration and activation of immune cells into the skin. Altogether our data provides important insights into how the loss of caspase-8 can have an impact beyond programmed cell death to affect the local microenvironment and elicit processes common to many neoplastic skin disorders.

654/B616

Molecular Mechanisms of Sexual Dimorphism in Chicken Feathers.

D. N. de la Cruz, J. Mayer, T. Jiang, C. Chuong, R. Widelitz; Pathology, University of Southern California, Los Angeles, CA

Secondary sexual characteristic are apparent throughout nature. Many of these characteristics can be observed as physical manifestations that differ between males and females. The primary objective of our study is to understand the role of sex hormones in the growth and development of sexually dimorphic skin appendages. The chicken model is a system that offers several sexually dimorphic skin appendages; furthermore tail feathers offer easily distinguishable sexual characteristics between male and female birds. There are seven pairs of tail feathers. In male chickens, tail feather lengths decrease bilaterally from the central aspect. This is in contrast to female tail plumage that consists of feathers that are of similar length. Our data indicate that male and female birds share similar structures; however hormone sensitivity confers increased size in male features. Differential length could be due to differences in growth rate or in the duration of the

growth period. We found that it is due to a combination of both factors with changes in the duration being the most significant. Consistent with this result, molecular expression revealed that proliferating cells are present in a higher concentration and for a longer period of time in males. It is our belief that the differential growth period is due to the influence of hormonal signaling. To this end, we have cloned the chicken androgen and estrogen receptors and performed in situ hybridization as well as immunostaining to localize them within the growing tail feather follicles. The pattern is distinct from that found in hair follicles. To further test hormone function, we manipulated hormonal signaling in the feather system by modulating levels of hormone receptors. This was accomplished using the RCAS retroviral system to deliver overexpression of full length receptors or by abating hormone signaling via the use of dominant negative receptors. The response of cell proliferation, apoptosis, signaling molecules, growth factors, adhesion molecules and differentiation markers were monitored to further explore the mechanism through which hormone stimulation may regulate the differential growth.

655/B617

Exposure to Microgravity during Spaceflight Reduced Cell Proliferation in the Regenerating Tail of The Newt Pleurodeles waltl.

N. Dvorochkin[1], R. Yousuf[2], E. A. Almeida[1,2]; [1]Space Biosciences Division, NASA Ames Research Center, Moffett Field, CA, [2]Department of Cell and Tissue Biology, University of California San Francisco, San Francisco, CA

Our working hypothesis is that spaceflight factors such as microgravity affect the growth and regenerative health of a broad range of tissues, including bone, muscle, and the immune system, by causing a slowing of normal cell proliferation needed for normal tissue regeneration. To test this broad hypothesis, we used the newt tail regeneration model and studied the effects of 12 and 16 day spaceflights on regenerative cell proliferation. To quantify cell proliferation in the newt tail we delivered water containing BrdU to the animals immediately before spaceflight, and subsequently performed BrdU immunolabeling and tissue morphometry. The experiment was performed during the US-Russian collaborative "Regeneration" experiments in the Foton M2 and M3 missions (total n=36). Our results show that regenerated tail length was significantly greater in ground controls that in flight, with 18% mean difference and p-value of 0.007. We also observed that the regenerated tail was bent downward to a greater extent in the ground group (63% mean difference, p-value of 0.0001). For quantifying BrdU incorporation in proliferating cells, we analyzed four different areas of the tail in histological sections: non-regenerated tail located proximally to the surgical cut, post-cut proximal regenerated tail, medial regenerated tail, and distal regenerated tail. Overall, proliferation in internal undifferentiated blastema tissue was greater then in differentiated epithelial cells of the regenerated tail. In epithelium, regeneration was the greatest in the area at the boundary with old tissue (~60%) and the lowest at the apex of the tail (~30%). Epithelial cell proliferation values were not statistically significant in flight versus ground controls. In the internal blastema tissue however, cell proliferation of ground controls was significantly greater than in all flight samples. Our results also show spaceflight altered the morphology of the regenerating tail, abolishing gravitropism of tissue regeneration at unit gravity, and decreasing cell proliferation in undifferentiated tail blastema tissue. Supported by NASA.

656/B618

Integrative Analysis of Cell and Tissue Behaviors during Eye Formation.

K. Kwan, H. Otsuna, C. Chien; Neurobiology and Anatomy, University of Utah, Salt Lake City, UT

The vertebrate eye develops as an evagination from the brain. This epithelium undergoes cell and tissue rearrangements to form initially the optic vesicle, and later, the optic cup. In zebrafish, this process occurs rapidly: evagination begins at 10 hours post fertilization (hpf), and the optic cup is fully formed by 24 hpf. We aim to develop a cellular and molecular understanding of the morphogenetic movements that generate the optic cup, using three levels of analysis: tissue movements; single cell behaviors and polarity; and molecular mechanisms controlling these movements. At the tissue level, we are performing whole-eye timelapse imaging throughout the entire period of eye morphogenesis; every cell is labeled for membranes and chromatin. Using these 4D data sets, we are generating a comprehensive map of cell movements and mitoses, and correlating this with changes in overall organ morphology. Initial analysis has revealed unexpected bulk flow of cells between certain subregions of the developing eye, and regionalized mitoses during different stages of morphogenesis. For single cell analysis, we utilize the photoactivatable fluorophore Kaede to label small groups of cells and observe single cell behaviors. Surprisingly, retinal progenitors display active lamellipodial protrusions, appearing migratory for most of morphogenesis; only ~20 hpf do they acquire their stereotypical columnar epithelial morphology. Because these cells make up an epithelial tissue, we examined cell polarity throughout eye morphogenesis. Strikingly, markers of apicobasal polarity are found asymmetrically localized as early as the onset of optic vesicle evagination; thus, the epithelium is polarized despite active motility of its component cells. To begin to uncover molecular pathways controlling eye morphogenesis, we are examining the role of laminin. We find that laminin is required for eye formation: in *bashful (laminin-α1)* mutants, the optic cup is disorganized, failure of optic vesicle invagination leads to a protruding lens phenotype, and apicobasal polarity is disrupted. We are now working to understand the mechanism by which laminin acts during eye morphogenesis, including identification of the cognate receptor.

Neuronal Diseases I (656A-675)

656A/B619

Evaluating Autophagic Regulation of Alpha-Synuclein in a Budding Yeast Model.

R. Choi, A. Konnikova, J. McLain, M. White, S. DebBurman; Lake Forest College, Lake Forest, IL

Parkinson disease (PD) is an incurable neurodegenerative disease that results from the selective loss of dopaminergic neurons in the substantia nigra. The misfolding and aggregation of the protein alpha-synuclein is the likely cause of cell death in PD. A popular hypothesis is that increasing the degradation of alpha-synuclein may protect the cell from its toxicity and aggregation. While the lysosome is pharmacologically implicated in the degradation of alpha-synuclein, the genetic link between PD and autophagy is less clear. A major route for delivering proteins to the lysosome is macroautophagy (hereafter referred to as autophagy). Autophagy is a highly conserved catabolic process in eukaryotes used to recycle and/or catabolize damaged or excess protein and organelles. We hypothesized that autophagy protects

cells from alpha-synuclein toxicity, and tested it by genetically inhibiting the expansion or nucleation steps within autophagy in an alpha-synuclein budding yeast model. We asked if alpha-synuclein would accumulate, relocalize and be more toxic in strains knocked out for autophagy genes (Atg) essential to those key steps. Thus far, in *atg17Δ* (needed for expansion), and *atg2Δ* and *atg18Δ* (needed for nucleation), the cellular localization of alpha-synuclein was subtly altered, but none induced toxicity. In *atg2Δ* yeast, alpha-synuclein aggregated more extensively while still maintaining its plasma membrane localization. While, in *atg17Δ* yeast, alpha-synuclein became more cytoplasmically diffuse and than plasma membrane localized. In these autophagy-deficient strains, alpha-synuclein did accumulate, but to varying extents. To more completely understand autophagy-mediated alpha-synuclein degradation, we are currently analyzing knockout strains for the rest of the proteins that comprise the expansion and nucleation steps of autophagy. (Supported by APDA, NSF-MRI, NSF-CCLI & NIH R15)

657/B620

Huntingtin Regulates Spindle Orientation During Mitosis.

K. Colombo-Maurat[1], B. Charrin[1], L. Nguyen[2,3], F. Guillemot[2], Y. Bellaiche[4], S. Humbert[1], F. Saudou[1]; [1]CNRS UMR 146, Institut Curie, Orsay, France, [2]Div Mol. Neurobiology, NIMR, Mill Hill, London, United Kingdom, [3]Developmental Neurobiology Unit, University of Liege, Liege, Belgium, [4]CNRS UMR144, Institut Curie, Paris, France

Huntingtin (htt) is a large protein of 350 kDa that when mutated causes Huntington's disease (HD), a neurodegenerative disorder characterized by severe psychiatric, cognitive and motor deficits and the selective death of neurons in the brain. The mechanisms that lead to disease are not fully understood but increasing evidence support that in addition to the gain of new toxic properties, loss of htt wild-type function participates to pathogenesis. Given the predominant neurological signs, most of the studies on htt function have focused on post-mitotic neurons. Using these approaches, important roles for htt in transcription and axonal transport have been elucidated but no studies have investigated the possible role of htt in dividing cells. Indeed, we and others have shown that wild-type htt interacts with the dynein/dynactin complex and kinesin to regulate the MT-dependent transport of organelles. Of interest, the proper integrity of MTs and of the dynein/dynactin complex is not only required for axonal transport in neurons but is also crucial for the correct positioning of the spindle during mitosis. Since htt facilitates dynein/dynactin transport of organelles along MTs in neurons and cells, we investigated whether htt could also function during mitosis. We show here that silencing htt in dividing cells leads to misorientated cell division and to an increased post-mitotic apoptosis. Htt localizes at the spindle poles from prophase to anaphase during mitosis and with dynactin at the cell cortex. Htt down-regulation by RNAi approaches leads to a spindle orientation defect reminiscent of the phenotype obtained when silencing p150Glued dynactin. We show that htt and p150Glued dynactin are interdependent as siRNA targeting htt mislocalizes p150Glued dynactin from the cell cortex to the spindle poles. Together, these results identify htt as a new regulator of spindle orientation in mammalian cells.

658/B621

HCMV UL97 Kinase Prevents Polyglutamine (polyQ) Aggresome Formation.

C. Tower[1], L. Fu[1], M. N. Prichard[2], E. Sztul[1]; [1]Cell Biology, UAB, Birmingham, AL, [2]Pediatrics, UAB, Birmingham, AL

A number of neurodegenerative diseases such as Huntington's (HD) and Spinocerebellar Ataxia-3 (SCA3) are caused by the expansion of a poly-glutamine (polyQ) tract within the mutant protein, huntingtin and ataxin-3, respectively. Poly-Q expansions are aggregation-prone and lead to the formation of cytoplasmic and nuclear aggregates that are hallmarks of these diseases. PolyQ aggregates disrupt cellular architecture, alter protein folding and degradation, and disregulate transcriptional activity, all of which contribute to neuronal toxicity. Inhibition of polyQ aggregation has been shown to prevent neurodegeneration in cellular, Drosophila, and mouse models of HD and SCA disease. We have previously shown that the UL97 kinase of the human Cytomegalovirus (HCMV) prevents the aggregation of viral tegument proteins in HCMV-infected cells. We hypothesized that UL97 may also be able to prevent the aggregation of polyQ proteins. To test this hypothesis, we examined whether UL97 can prevent the aggregation of mutant htt and ataxin-3 constructs containing expanded polyQ tracks. We show that UL97 prevents aggregation of httex1-82Q-YFP as well as the ataxin3-72Q-myc. Kinase activity of UL97 is required, since a catalytically inactive mutant of UL97 fails to disperse the aggregates. UL97 localizes to the nucleus and appears to disrupt the architecture of PML bodies. PML bodies have been implicated in transcriptional function as they contain such transcriptional regulators as Sp100, PML, CBP and p53. We propose that UL97 may inhibit aggregation by upregulating the transcription of proteins that aid in protein folding and/or clearance.

659/B622

Strategies for the Identification and Validation of Huntington's Disease Therapeutic Targets.

D. Marchionini[1], S. Kwak[2], D. Howland[2], J. Wang[1], E. Signer[1]; [1]CHDI Management, New York, NY, [2]CHDI Management, Princeton, NJ

During the past six years, CHDI Foundation supported projects to identify and evaluate potential new therapeutic targets using a target-validation (TV) scale modeled on those of several pharmaceutical companies. We systematically assessed more than 300 potential targets and assigned TV scores, based on published results and on new studies specifically directed toward target molecules. Assigned TV scores, which classify targets according to their potential utility for Huntington's Disease (HD) therapies, range from 0 (no known link to HD), through 1 and 2 (target identification), 2.5 and 3.5 (in vitro and in vivo validation in models of HD), 4.0 and 4.5 (pharmacological or gene therapy intervention, or Phase 2 clinical trial), and 5.0 (Phase 3 trial). Of the 338 gene products so far evaluated, 9% have TV scores of 3.5 or more, and will be presented. Collaborations with academic laboratories and contract research organizations, and data from cell-based loss-of-function and overexpression assays, interactome studies and microarrays, are expanding the list of potential targets. We also established an Early Discovery Initiative to encourage pilot projects to identify novel targets and approaches. This initiative supports novel, high-risk ideas, especially from investigators new to HD research. To assist in the evaluation of targets, we are developing a pathway analysis database to incorporate new and old data as well as information from publically available databases and the Ingenuity Pathway Analysis. We will continue to prosecute already identified targets (TV > 3.5), but there is still a great need for novel targets and pathways that may lead to a therapy for HD.

660/B623

Oligomeric Cylindrical Structure of Expanded Polyglutamine Protein Is Cytotoxic.

S. Katada[1], T. Takahashi[1,2], M. Nishizawa[1], O. Onodera[3]; [1]Neurology, Brain Research Institute, Niigata University, Niigata, Japan, [2]Health Science, Faculty of Medicine, Niigata University, Niigata, Japan, [3]Molecular Biology, Brain Research Institute, Niigata University, Niigata, Japan

Expanded polyglutamine (polyQ) protein causes neurodegenerative diseases in a length-dependent manner. The previous studies have indicated that the conformational change of expanded polyQ proteins underlies the pathogenesis of polyQ diseases. Non-toxic expanded polyQ monomers will transform into toxic monomers, and then assemble into oligomers, protofibrils and inclusion bodies(IBs). Although IBs were suspected to be toxic, several lines of evidence have indicated that the structures preceding the formation of IBs are more cytotoxic. We have recently shown that the oligomerization of polyQ associates with cytotoxicity in neuronal cells by using fluorescence resonance energy transfer (FRET) microscopy. We proposed a model for polyQ dynamics: polyQ monomers assemble into oligomers in a parallel orientation or in a head-to-tail orientation with cylindrical structure. However, it is still unclear whether the formation of oligomeric structure of polyQ is necessary to produce cytotoxicity. To investigate 1)oligomeric structure of polyQ in living cells and 2)toxicity of polyQ monomers, we applied FRET assay to truncated DRPLA proteins tagged with fluorescent proteins at both terminals (mCFP-trDQ-mCFP, mYFP-trDQ-mYFP, and mCFP-trDQ-mYFP). FRET-positive cells were only observed in cells transfected with mCFP-trDQ-mYFP, not the cells cotransfected with mCFP-trDQ-mCFP and mYFP-trDQ-mYFP. These findings indicate that the non-toxic expanded polyQ monomers transform into cylindrical structure. Furthermore no IBs were observed in the cells transfected with mCFP-trDQ-mYFP, while these cells are FRET positive, suggesting that mCFP-trDQ-mYFP hold monomeric state with cylindrical conformation. To investigate the toxicity of monomeric cylindrical polyQ proteins, we performed cell survival assay by using the neuronally differentiated SH-SY5Y cells which are stably expressing mYFP-trDQ58-mYFP (monomeric construct) or oligomeric constructs (trDQ58-mYFP). The cells expressing monomer constructs were more viable compared with the cells expressing oligomeric constructs. On the basis of these finding, we conclude that expanded polyQ forms cylindrical structure and oligomeric formation of polyQ take an important role for cytotoxicity.

661/B624

Development of a Novel Model for Polyglutamine Pathogenesis Using the Chicken Embryo.

H. Nakayama[1], M. Shibata[1], T. Takahashi[2], O. Onodera[3], N. Sato[1]; [1]Division of Gross Anatomy and Morphogenesis, Niigata University, Graduate School of Medical and Dental Sciences, Niigata-city, Japan, [2]School of Health Sciences, Niigata University, Faculty of Medicine, Niigata-city, Japan, [3]Department of Molecular Neuroscience, Niigata University, Brain Research Institute, Niigata-city, Japan

Polyglutamine (polyQ) diseases are dominantly inherited neurodegenerative disorders caused by the expansion of glutamine-encoding repeat within each identified genes. Accumulating evidences suggest that gain-of-function of polyQ proteins, but not loss-of-function of naïve proteins, leads to neurodegeneration. To study polyQ pathogenesis in vivo we took advantage of the chicken embryo system which now allows efficient gene transfer into the developing tissues including the nervous system. In the present study, we confirmed the polyQ proteins are introduced into the neural tube efficiently 24 hrs after transfection of polyQ constructs by in ovo electroporation. Transfer of a truncated atrophin-1 gene carrying 80 polyQ repeat (trD-Q80) into the developing spinal cord resulted in appearance of numerous cells expressing diffuse trD-Q80. In addition, cells formed aggregates of trD-80 in the nucleus as well as in the cytoplasm. In polyQ-transferred sides of the spinal cord, cells appeared to undergo cell death judged by the TUNEL staining and the activated caspase-3 immunoreactivity. Moreover, aggregated ubiquitin immunoreactivity was observed in the polyQ-transferred spinal cord. Taken together, these results suggest that misexpression of polyQ protein in the chicken embryo spinal cord can be an alternative model for investigating cellular pathogenesis of polyQ proteins in vivo.

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Mechanisms of Polyglutamine Aggregate Transmission in Huntington's Disease.

J. E. Lauckner[1], R. Melki[2], R. R. Kopito[1]; [1]Biology, Stanford University, Stanford, CA, [2]Laboratoire d'Enzymologie et Biochimie Structurales, Centre National de la Recherche Scientifique, Gif-sur-Yvette, France

Huntington's disease (HD) is a dominantly inherited autosomal disorder caused by a CAG expansion in the ubiquitous huntingtin protein. Although HD is purely genetic in transmission between individuals, it is not known whether the aggregation phenotype and pathology can be transmitted between neurons in the brain. Our lab has recently found that exogenously applied fibrillar polyQ$_{44}$ aggregates bind tightly to, and are internalized by, mammalian cells. These internalized fibrillar aggregates can recruit and nucleate soluble, short polyglutamine reporter proteins and can propagate the phenotype in a heritable, non-genetic manner through multiple generations. It is possible that the infectious transmission of these polyQ aggregates contributes to the intracellular transmission of HD. We have studied the mechanisms of cytoplasmic entry of polyQ$_{44}$ aggregates. Using flow cytometry, fluorescent and electron microscopy, and chemical modification of fluorescently-tagged aggregates, we have found that aggregate entry is likely a multi-step process including sequential binding to the cell surface, permeation of the plasma membrane, and entry into the cytoplasm. Aggregate entry increases membrane permeability to propidium iodide and dextrans suggesting the production of aggregate-induced membrane pores. This membrane permeability is transient-suggesting the existence of membrane repair mechanisms. Once internalized, polyQ$_{44}$ aggregates can cause aggregation of soluble polyQ$_{25}$. Synthetic, bacterially-produced, and biologically-expressed aggregates all have similar effects, however we have found that aggregate structure must be fibrillar and β-sheet-rich to enable penetration. Taken together, our data indicate that fibrillar polyQ aggregates are candidates to be involved in cell-to-cell disease transmission and non-cell autonomous neurotoxicity.

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Soluble Polyglutamine Oligomers Formed Prior to Inclusion Body Formation are Cytotoxic.

T. Takahashi[1,2], S. Kikuchi[2], S. Katada[2], M. Nishizawa[2], O. Onodera[3]; [1]Health Science, Faculty of Medicine, Niigata University, Niigata, Japan, [2]Neurology, Brain Research Institute, Niigata University, Niigata, Japan, [3]Molecular Biology, Brain Research Institute, Niigata University, Niigata, Japan

Expanded polyglutamine repeats cause neurodegenerative disorders, but their cytotoxic structures remain to be elucidated. Although soluble polyglutamine oligomers have been proposed as a cytotoxic structure, the cytotoxicity of soluble polyglutamine oligomers, not inclusion bodies (IBs), has not been proven in living cells. To identify soluble polyglutamine oligomers in living cell and investigate the cytotoxicity, we constructed fusion protein of a truncated atrophin-1 or huntingtin exon1 containing various lengths of polyglutamine repeats with monomeric variants of CFP (mCFP) or YFP (mYFP) [trD-Qn-mCFP/mYFP, Htt-Qn-mCFP/mYFP] and cotransfected these constructs into COS7 cells in various combinations, and then carried our fluorescence resonance energy transfer (FRET) confocal microscopy. FRET signals were detected when donor and acceptor fluorescence proteins were attached to the same side, not the opposite side, of polyglutamine repeats, which agrees with a parallelβ-sheet or a head-to-tail cylindrical β-sheet model. Polyglutamine monomers assembled into soluble oligomers in a length-dependent manner, which was followed by the formation of IBs. Furthermore, we showed the oligomer was soluble and dissociate into monomer in semi intact cell. Time-lapse imaging revealed that transition from the soluble oligomers to the insoluble IBs is rapid accompanied by a reduction in the amount of soluble oligomers. To determine the relationship between soluble oligomer formation and the risk of cell death, we established stable cell lines after transduction of lentiviral vectors containing trD-Q75-mCFP/mYFP to SH-SY5Y cells. Then we compared the survival curves of neuronally differentiated SH-SY5Y cells that did or did not develop detectable soluble oligomers. Notably, survival assay of neuronally differentiated cells revealed that cells with soluble oligomers died faster than those with IBs or monomers. IBs formation may sequestrate the toxic polyglutamine structures and protect cells from the cytotoxity of expanded polyglutamine repeats. These results indicate that a length-dependent formation of oligomers is an essential mechanism underlying neurodegeneration in polyglutamine-mediated disorders.

664/B627

The Role of VPS41, a Protein Involved in Lysosomal Trafficking, in C. Elegans and Mammalian Models of Parkinson's Disease.

A. J. Harrington[1], Q. Ruan[2], S. Hamamichi[1], D. G. Standaert[2], K. A. Caldwell[1,2], G. Caldwell[1,2]; [1]Biological Sciences, University of Alabama, Tuscaloosa, AL, [2]Dept of Neurology, University of Alabama School of Medicine, Birmingham, AL

Parkinson's disease (PD) is the second most common neurodegenerative disorder. Studies of familial forms of PD have identified genes that when mutated or over-expressed, resulted in enhanced susceptibility of dopamine (DA) neurodegeneration. One of these proteins, alpha-synuclein (α-syn), has been shown to aggregate and form protein inclusion bodies. Accumulation of GFP-tagged human α-syn can be observed in *C. elegans*, where these transgenic worms were used to screen for effectors of age-dependent aggregrate formation *in vivo*. Using RNAi, our lab has identified several genes that, when knocked down, increase aggregate formation within the body wall muscles. Notably, overexpressing several of these effector proteins in worm DA neurons led to protection from α-syn-induced neurodegeneration. Of these neuroprotective proteins, a lysosomal trafficking protein, VPS41, was the most effective at preventing neurodegeneration. VPS41 is a highly conserved protein expressed in mammalian DA neurons. It has metal ion binding, microtubule binding, RING finger, and AP3 interaction domains, and in yeast is involved in lysosomal trafficking. To identify the domains of VPS41 responsible for its protective actions, we truncated different domains of human VPS41 and are over-expressing these truncates in α-syn-expressing DA neurons in worms. Several isoforms of VPS41 have shown protection against α-syn induced neurodegeneration. Importantly, the neuroprotective capacity of VPS41 extends to mammalian cellular models where SH-SY5Y neuroblastoma cells transfected with human VPS41 exhibit protection from a broad kinase inhibitor, staurosporine, and the PD-related neurotoxin, rotenone. The presence of VPS41 in these cells significantly reduces the sensitivity of the lines to both toxins. These data demonstrate that VPS41 is part of an evolutionarily conserved mechanism that is neuroprotective in both mammalian cells and *C. elegans*. Thus, the VPS41 protein and, by extension, the regulation of autophagy, represents a promising target for therapeutic development against PD.

665/B628

Investigating the Neuroprotective Role of miRNAs in a C. Elegans Model of Parkinson's Disease.

S. M. DeLeon, S. Hamamichi, K. A. Caldwell, G. A. Caldwell; Biological Sciences, University of Alabama, Tuscaloosa, AL

Parkinson's disease (PD) is the second most common neurological disorder. Studying familial forms of PD has revealed that mutation or multiplication of the α-synuclein (α-syn) gene contributes to α-syn misfolding and aggregation, as well as subsequent progressive degeneration of dopamine (DA) neurons. We previously generated an isogenic line of the nematode, *C. elegans*, overexpressing human α-syn fused to GFP in worm body wall muscle cells to conduct systematic RNA interference (RNAi) screen of nearly 900 targets, and reported that 20 genetic candidates, when knocked down in a background that attenuated protein misfolding, visibly enhanced α-syn::GFP aggregation. Previously, using transgenic worms that overexpress α-syn in the DA neurons, we showed age-dependent neurodegeneration could be rescued by the conserved intracellular trafficking proteins of the Rab family. Likewise, we also determined that 5 out of 7 candidates examined from our RNAi screen subsequently protected DA neurons from α-syn induced toxicity when overexpressed. Given the regulatory role of microRNAs (miRNAs) in global gene expression, we conducted a bioinformatic analysis to identify miRNAs that are predicted to target multiple neuroprotective genes as determined by our model. To test if these miRNAs function to co-regulate specific targets in networks that can attenuate α-syn toxicity and cell death, we performed genetic crosses between the isogenic line of worm overexpressing α-syn (and GFP as a marker) in *C. elegans* DA neurons and miRNA loss-of-function mutant strains. Presently, we have identified one miRNA that, when knocked-out, significantly rescues α-syn induced neurodegeneration. Further analysis is underway to determine if additional miRNAs predicted to regulate neuroprotective genes may function in the same capacity. The identification of specific regulatory miRNAs that function to mediate neuronal survival would represent a novel network of targets for functional validation in mammals. Taken together, our approach highlights the strengths of the nematode system to functionally delineate modifiers of cellular processes that mediate neuroprotection and accelerate the translational path toward therapeutic development.

666/B629

Regulation of Parkinson's Disease Protein DJ-1 Protease Activity and Cytoprotective Function.

J. Chen, L. Chin, L. Li; Pharmacology, Emory University, Atlanta, GA

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, characterized by degeneration of dopaminergic neurons in the substantia nigra. Recent identification of mutations in DJ-1, a ubiquitously expressed protein of unknown function, as the cause for an autosomal recessive, early-onset form of PD provides strong evidence linking the loss of DJ-1 function to neurodegeneration. To gain insight into DJ-1 function, we and others have solved the DJ-1 crystal structure, which reveals that DJ-1 shares high degree of structural homology with the PfpI family of intracellular proteases. However, DJ-1 has an additional C-terminal alpha-helix, H8, which may block the access of its substrate to the catalytic site. Consistent with the structural data, our biochemical analyses show that recombinant full-length DJ-1 protein possesses intrinsic cysteine protease activity in vitro and the DJ-1 protease activity is significantly increased by the deletion of DJ-1 H8 helix. Moreover, deletion of DJ-1 H8 helix also enhances the cytoprotective action of DJ-1 against oxidative stress-induced cell death. Our findings suggest that the C-terminal tail of DJ-1 plays a key regulatory role in governing DJ-1 protease activity and cytoprotective function and provide new insights into the pathogenic mechanisms of DJ-1 mutations in PD.

667/B630

Dopamine Signal Transduction Components Alter Neurodegeneration in a Parkinson's Disease Model.

L. Berkowitz, S. Hamamichi, K. A. Caldwell, G. Caldwell; Biological Sciences, University of Alabama, Tuscaloosa, AL

Improvements to the diagnosis and treatment of Parkinson's disease (PD) are contingent upon knowledge about susceptibility factors that render populations at risk. Our work has focused on a functional analysis that exploits the experimental attributes of the nematode *Caenorhabditis elegans* to accelerate the translational path toward identification of effectors of dopamine (DA) neuron degeneration *in vivo*. *C. elegans* has only 8 DA neurons, providing a simple system in which to study PD-like neurodegeneration with unparalleled accuracy in quantitation of neuron survival in statistically substantial numbers of test animals. Our prior application of *C. elegans* in this directed manner has been successful in rapidly predicting genes that have significant consequences for neuronal survival in mammalian systems (Cooper et al., 2006, *Science*; Gitler et al., 2008, *PNAS*). Human α-synuclein, a protein found in the PD-associated aggregates called Lewy bodies, when overexpressed in *C. elegans* DA neurons causes them to degenerate in an age- and dose-dependent manner. The GAIP Interacting Protein C-terminus (GIPC) was identified in a large-scale RNAi screen for genes that alter both α-synuclein aggregation and DA neuron degeneration (Hamamichi et al., 2008, *PNAS*). GIPC has been proposed to play multiple roles in DA signal transduction: 1) membrane recruitment of a GTPase Activating Protein (GAP, which down-regulates DA signaling), 2) DA receptor internalization, 3) DA receptor recycling. Here we report that genetic loss of GIPC enhances neurodegeneration, whereas overexpression is neuroprotective. To determine what GIPC-dependent function(s) is involved in its neuroprotective capacity, proteins that act in the above pathways are being analyzed via genetic knockouts and DA neuron-specific overexpression. Loss of presynaptic dopamine D2 receptor increases degeneration. Loss of the *C. elegans* GAP, *rgs-2*, is neuroprotective while overexpression enhances DA neuron loss. Together these results suggest that components of the DA signaling cascade contribute to neuron survival.

668/B631

Neuroprotective Role of UNC-51/ULK2 in a C. Elegans Model of Parkinson's Disease.

M. A. Thompson, A. J. Harrington, S. Hamamichi, K. A. Caldwell, G. Caldwell; Biological Sciences, University of Alabama, Tuscaloosa, AL

Pathological features of Parkinson's disease (PD) include the accumulation and aggregation of α-synuclein (α-syn) into inclusions called Lewy bodies, along with progressive loss of dopamine (DA) neurons. Recent advances in understanding neuroprotective mechanisms against α-syn induced toxicity have revealed autophagy as a critical pathway involved in the degradation of α-syn that also enhances DA neuron survival. Autophagy, which is a conserved pathway across the species, is a membrane-mediated means of targeted intracellular transport to lysosomes for degradation of misfolded proteins as well as organelles. One of the autophagic components is UNC-51/ULK2 (UNC-51-like kinase 2), which has been shown to localize to the isolation membrane, and its kinase activity is required for autophagosome formation. Previously, we reported the results from a large-scale systematic RNA interference (RNAi) screen, conducted in *C. elegans* , wherein RNAi knock-down of *T07F12.4* (serine/threonine kinase similar to *unc-51/ULK2*), *vps-41/VPS41*, and *atgr-7/ATG7* all enhanced α-syn misfolding, thereby demonstrating an inherently protective role of these autophagy-associated proteins in α-syn misfolding. Given the identification of *ULK2* as one of the 6 genes modified in genome-wide analysis of single nucleotide polymorphisms (SNPs) in PD patients, we examined the neuroprotective capacity of UNC-51/ULK2. We have previously generated an isogenic line of *C. elegans* overexpressing α-syn and GFP in the DA neurons, which displays dose- and age-dependent neurodegeneration. Using these animals, we have performed genetic crosses to generate strains for analysis of α-syn induced neurodegeneration in either a *unc-51* loss-of-function mutant background or in animals overexpressing *ULK2* in the DA neurons. Our data indicate while *unc-51 loss*-of-function mutation enhanced α-syn induced toxicity, overexpression of *ULK2* protected the DA neurons. These combined results strongly support autophagy as a neuroprotective cellular pathway, and implicate enhanced activity of ULK2 as a possible therapeutic target for treatment of PD.

669/B632

Development of a hESC Model System to Examine Processing and Secretion of Amyloid Beta in Human Neurons.

E. Davis[1], J. D. Flippin[1], N. Jeon[2], L. Goldstein[1]; [1]Cellular & Molecular Medicine, University of California San Diego, La Jolla, CA, [2]Biomedical Engineering, University of California Irvine, Irvine, CA

Alzheimer's disease (AD) is a neurodegenerative disease characterized by amyloid plaques, neurofibrillary tangles and a massive loss of neurons in affected brains. The major constituent of amyloid plaques is extracellular Amyloid beta (Abeta) peptides, which are processed from beta-Amyloid Precursor Protein (APP) by gamma- and beta-secretases. APP is transported on vesicles in axons, and is also involved in regulation of axonal transport. The localization of APP processing as well as the details of Abeta secretion have yet to be fully elucidated. To date, the majority of this work has been performed in non-human cells, and the details in human neurons remain unclear. We have developed a system using neurons derived from human embryonic stem cells (hESCs) in a novel microfluidic culture device. This device allows us to assess the biochemistry of APP and Abeta in axons independent of the soma in these neurons. This, as well as the development of additional

assays, will help determine the levels of human endogenous axonal APP processing and Aβ secretion, as well as from disease-model cell lines.

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Amyloid Beta Induced Signaling - Molecular Events Preceding Cell Death.

J. Reifert, D. Hartung, S. Feinstein; MDCB - NRI, UCSB, Santa Barbara, CA

Alzheimer's disease is characterized clinically by the accumulation of amyloid beta (Aβ) peptides into plaques, aggregation of the microtubule associated protein tau into neurofibrillary tangles (NFT's) and neuronal cell death. One model attempting to connect these events is the amyloid cascade hypothesis, which suggests that soluble Aβ oligomers alter cellular processes resulting in aberrant tau phosphorylation and truncation, ultimately leading to the production of NFT's and neuronal cell death. However, the role of NFT's themselves (as opposed to dysfunctional tau) in the cell death process remains uncertain. In order to identify molecular "players" affected by exposure to Aβ that may promote pathological tau action, cultured rat hippocampal neurons were treated with oligomerized Aβ and a timecourse of biochemical samples were harvested prior to detectable levels of cell death. Multiple signaling pathways were evaluated for dysregulation via western blotting using antibodies specific for active versus inactive signaling elements. Protease activities were measured using activity assays and western blot imaging of substrate cleavage. Hippocampal neurons exposed to Aβ results in rapid activation of both caspase and calpain protease activities. This is followed by reduced levels of active AKT and increased levels of activated GSK3β. Shortly thereafter, we observed tau degradation. These events precede cell death as measured by both metabolic functioning and membrane integrity of the neurons. Taken together, these data are consistent with the view that Ab induces a rapid increase in calpain and caspase activities which could serve to degrade tau. On a slightly slower but still relevant time scale, Aβ may also influence tau phosphorylation through downregulation of the AKT signaling pathway resulting in increased active GSK3β, a known kinase targeting tau. Because all these processes precedes cell death, the data highlight the potential importance of tau proteolysis and phosphorylation in pathological tau action.

671/B634

Small β-Amyloid Oligomers Induce Tau-Dependent Microtubule Disassembly: a Primary Cause of Synapse Loss in Alzheimer's Disease?

M. E. Seward[1], M. Jin[2], M. E. King[1], D. J. Selkoe[2], G. S. Bloom[1]; [1]Depts of Biology, and Cell Biology, University of Virginia, Charlottesville, VA, [2]Center for Neurologic Diseases, Brigham and Women's Hospital, and Harvard Medical School, Boston, MA

Alzheimer's disease (AD) symptoms are caused by reduced synaptic function, and are associated with accumulation of amyloid plaques and neurofibrillary tangles in brain. Plaques and tangles differ in molecular composition, but their dominant structural features are insoluble, fibrous polymers. Plaques comprise β-amyloid (Aβ) peptide fibrils, whereas tangles contain filaments formed by the neuronal microtubule (MT) associated protein, tau. A key unresolved issue about AD is how Aβ, tau, and the abnormal polymers they form contribute to synaptic dysfunction. It was recently demonstrated in cultured cell models that synthetic, pre-fibrillar Aβ induced tau-dependent MT loss, which was hypothesized to compromise MT-dependent axonal transport in vivo, and by extension, delivery to axon terminals of proteins essential for synaptic function (King, et al. 2006. J CELL BIOL 175: 541-546). Pre-fibrillar Aβ contained a complex mix of monomers and variably sized oligomers, and was active at ~1 μM total peptide, prompting the question of which Aβ species induced MT loss. To address this question, we assayed Aβ secreted by 7PA2 cells, a Chinese hamster ovary (CHO) cell line that stably expresses a mutant human β-amyloid precursor protein and overproduces Aβ (Podlisny, et al. 1996. J BIOL CHEM 270: 9564-9570). We now demonstrate that apparent dimers, trimers and tetramers, but not monomers of this biologically produced Aβ induce tau-dependent MT dissassembly at ~1-10 nM total peptide. Treatment of tau-expressing CV-1 cells with dimer/trimer and tetramer fractions depolymerized MTs within 90 minutes. MT loss was not observed in tau-positive CV-1 cells exposed to Aβ monomers or a dimer/trimer-equivalent fraction secreted by wild type CHO cells that do not overproduce Aβ. Furthermore, MTs in tau-negative CV-1 cells were resistant to Aβ dimers/trimers and tetramers. In a related study of CV-1 cells expressing small tau fragments, two regions in the N-terminal half of tau were found to confer sensitivity of MTs to small Aβ oligomers. Together, these results raise the possibility that tau N-terminal-dependent MT loss triggered by trace levels of very small Aβ oligomers is a primary cause of synaptic failure in AD.

672/B635

RING Finger Protein 11 is a Novel E3 Ligase that Ubiquitinates Mitochondrial Proteins.

B. Whatley, L. Chin, L. Li; Pharmacology, Emory University, Atlanta, GA

Neurons affected by neurodegenerative disease exhibit dysfunction of both mitochondria and the ubiquitin system, suggesting a functional link between the two. Understanding the interplay between these two systems is crucial for comprehending the molecular mechanisms of disease progression. Although there is evidence that mitochondrial proteins are ubiquitinated, little is known about specific mitochondrial E3 ubiquitin-protein ligases and few substrates of these E3 ligases have been identified. Of the molecular components that comprise the ubiquitin system, the E3 ligase is perhaps the most important player because it determines the specificity of ubiquitination. RING finger protein 11 (RNF11) is a neuronal protein that contains a RING finger, a motif that has been implicated as a key determinant of E3 ligase activity. The biochemical function and cellular role of RNF11 remain poorly characterized. To understand the function of RNF11 in the cell, we wanted to identify substrates for RNF11-mediated ubiquitination and thereby discover cell processes that may be impacted by RNF11. Using yeast two-hybrid screening, we identified two mitochondrial interactors of RNF11. We confirmed that a population of RNF11 localizes to mitochondria by both immunofluorescence confocal microscopy and subcellular fractionation. Using both in vitro and in vivo ubiquitination assays, we have determined that RNF11 is capable of ubiquitinating mitochondrial proteins. These results suggest that RNF11 E3 ligase may play a role in the regulation of mitochondrial function.

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The E3 Ubiquitin Ligase Mahogunin Ring Finger-1 Regulates Cellular Hook1 Levels and Mediates its Association with Rab7.

J. Jiao, T. M. Gunn; Biomedical Sciences, Cornell University, Ithaca, NY

Spongiform neurodegeneration is characterized by the appearance of vacuoles throughout the central nervous system (CNS). It has many potential causes but the underlying cellular mechanisms are not well understood. Mice lacking the E3 ubiquitin ligase Mahogunin Ring Finger-1 (MGRN1) develop age-dependent spongiform encephalopathy. We hypothesize that loss of MGRN1-mediated ubiquitination of specific proteins leads to vacuolation of the CNS. Using yeast two hybrid and coimmunoprecipitation assays, we identified an association between endogenous MGRN1, Rab7 and Hook1, a microtubule binding protein that is thought to link endocytic membrane trafficking to the microtubule cytoskeleton. Ubiquitination assays in HEK293T cells demonstrated that MGRN1 regulates cellular Hook1 levels by targeting it for ubiquitin-mediated degradation. Over-expression of MGRN1 resulted in less Rab7-bound Hook1, while over-expression of catalytically inactive MGRN1 increased the association of Hook1 with Rab7. We propose a model in which MGRN1-mediated ubiquitination facilitates the dissociation of Hook1 from Rab7 to allow trafficking of multivesicular bodies (MVBs) to lysosomes. This is consistent with observations in Drosophila, where reduced Hook1 levels were associated with an increased rate of MVB trafficking (Sunio et al., 1999). Loss of MGRN1 results in increased Hook1 levels, which would be expected to reduce the rate of MVB trafficking to the lysosome, and the resulting accumulation of MVBs could represent a mechanism for the development of vacuoles within cells.

674/B637

Impairment of the Ubiquitin Proteasome System by Aggregation Prone Proteins Is Independent of Ubiquitylation.

M. S. Hipp[1], E. J. Bennett[2], R. R. Kopito[1]; [1]Department of Biological Sciences, Bio-X Program, Stanford University, Stanford, CA, [2]Pathology Department, Harvard Medical School, Boston, MA

The presence of aggregated proteins is a hallmark for nearly all neurodegenerative diseases. However, the mechanism for, and the target of aggregate toxicity is still unknown. We have previously shown that one target of aggregated proteins is the ubiquitin proteasome system (UPS) (Bence et al. Science. 2001 May 25;292(5521):1552-52001, Bennett et al. Nature. 2007 Aug 9;448(7154):704-8). The UPS controls the stability of most proteins and is therefore essential for virtually all aspects of cellular function. It is composed of several hundred proteins. Among these are ubiquitin itself, a huge number of enzymes involved in the conjugation of ubiquitin to a target protein, a smaller number of deconjugating enzymes, ubiquitin binding proteins, and the 26S proteasome, a multi-subunit protease. Using various UPS reporter substrates, we report that the target of aggregated proteins is down-stream of the ubiquitylation cascade, and that aggregated proteins inhibit a general component of the UPS and not only a specialized subset of it.

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Investigation of a Monocarboxylate Shuttle System in the Mammalian Retina.

J. D. Ochrietor, A. McCormack, M. Zahra, B. Yaqubi, G. Wilhoite, N. Finch; Biology, University of North Florida, Jacksonville, FL

Basigin gene products are members of the immunoglobulin superfamily and are thought to mediate cell adhesion events. In the retina, two Basigin gene products are expressed. Basigin is found on the surface of Müller cells, whereas Basigin-2 is found on the surface of photoreceptors. Mice in which the Basigin gene is deleted are characterized as having severely depressed electroretinograms from the time of eye opening, which suggests blindness in these animals. Biochemical and immunohistological analyses of Basigin null mice indicate that Monocarboxylate transporter 1 (MCT1) expression is also altered. We have proposed that a monocarboxylate shuttle complex exists within the retina, such that Basigin and MCT1 on Müller cells and Basigin-2 and MCT1 photoreceptors coordinate transfer of lactate, pyruvate, or ketone bodies (all monocarboxylates) from the Müller cells to the photoreceptors. The photoreceptors then use the monocarboxylate metabolite as fuel for oxidative phosphorylation. We hypothesize that the lack of a monocarboxylate shuttle system is the molecular basis for blindness in Basigin null mice. Therefore, the purpose of this study was to examine the molecular interactions governing monocarboxylate shuttle assembly in the mammalian retina. Recombinant and endogenous versions of Basigin, Basigin-2, and MCT1 were used in enzyme-linked immunosorbant assays (ELISA) to study interactions between the shuttle components. It was determined that Basigin gene products bind MCT1 via hydrophobic interactions between regions flanking the plasma membrane. It was also found that the Basigin gene products interact via their extracellular domains. In addition, it was determined that cyclophilin A (CyPA), a molecular chaperone, also interacts with both Basigin gene products and MCT1. These data suggest that interactions between Basigin gene products and MCT1 mediate complex formation on Müller cells and photoreceptors. The two complexes are then connected via the extracellular domains of Basigin gene products, to adhere the two cell types. It is thought that CyPA mediates translocation of each complex to its respective plasma membrane; however, additional experiments must be performed to confirm this.

Host-Pathogen Interactions: Bacteria

676/B639

Tight Junction Dysfunction Promotes Adhesion and Invasion of H. Hepaticus in Polarized Colon Epithelial Cells.

K. Mair[1], C. Beckwith[2], M. Amieva[3], R. M. Schmid[1], R. Vogelmann[1]; [1]Internal Medicine II, Technical University Munich, Munich, Germany, [2]Comparative Medicine, Hershey Medical Center, Hershey, PA, [3]Microbiology & Immunology, Stanford University, Stanford, CA

Objective: Intestinal epithelial cells (IEC) form a barrier to prevent microbial factors from entering host organisms. A defective barrier exposes non-invasive facultative pathogens to the immune system contributing to inflammation, which can manifest as Inflammatory Bowel Disease (IBD) in humans. Bacterial adhesion to and invasion of IEC is increased in IBD or IBD-like mouse models. We tested if a barrier defect of IEC promotes adhesion and invasion of an otherwise non-invasive facultative pathogen *Helicobacter hepaticus*. Methods: We infected polarized epithelial cells (MDCK or PTK6 cells) grown on Transwell filters with *H. hepaticus*. We compared apical and basal-lateral infections with or without opening of the barrier via previous Ca^{2+} depletion. Results: In polarized PTK6 cells (mouse colon cell line) 24 (SE ± 9) bacteria adhere after 18 h when infected from apical with intact barrier. In contrast, in apical infections with an open barrier or basal-lateral infections 133 (SE ± 27) or 112 (SE ± 27) bacteria/100 IEC adhered after 18 h, respectively. Adhesion was followed by bacterial invasion 2 h after begin of infection. Eighteen hours later, 70 (SE ± 5) bacteria/100 IEC were intracellular when infected apical with an open barrier. Surprisingly, when infected from basal-lateral only 9 (SE ± 1) bacteria/100 IEC were found intracellular when the barrier remained

intact indicating that an open barrier facilitates invasion of basal/lateral attached bacteria. A gentamycin protection assay showed that *H. hepaticus* was not able to grow in media containing gentamycin. However, in the presence of non-confluent PTK6 cells the amount of *H. hepaticus* specific DNA quantified by qPCR increased by $4.3x10^4$ -fold in the supernatant during a 24 h course of gentamycin treatment. Conclusions: Our results show for the first time that *H. hepaticus* preferentially adheres to the basal-lateral membrane of polarized IEC and invasion depends on tight junction dysfunction. *H. hepaticus* is able to replicate intracellular and the data suggest that barrier dysfunction may play an important role for *H. hepaticus* mouse colonization hence colon inflammation.

677/B640

SopB Prevents Salmonella-Containing Vacuole Fusion with Lysosomes by Controlling Membrane Charge.

M. Bakowski[1,2], G. Lam[1,3], V. Braun[1], T. Yeung[1], W. Heo[4], B. Finlay[5], T. Meyer[4], S. Grinstein[1], J. Brumell[1,2,3]; [1]Program in Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada, [2]Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada, [3]Institute of Medical Science, University of Toronto, Toronto, ON, Canada, [4]Department of Molecular Pharmacology, Stanford University Medical School, Stanford, CA, [5]Michael Smith Laboratories, University of British Columbia, Vancouver, BC, Canada

Salmonella enterica serovar Typhimurium (*S.* Typhimurium) is a facultative intracellular Gram-negative pathogen that causes disease in diverse hosts. These bacteria are able to establish a replicative niche within host cells in the *Salmonella*-containing vacuole (SCV) by avoiding fusion with lysosomes. Bacterial type III secreted effectors are known to alter SCV trafficking in host cells but the precise mechanism(s) of lysosomal avoidance by *S.* Typhimurium are not well understood. SopB is a type III secreted effector with lipid phosphatase activity. It contributes to bacterial invasion by modifying the lipid composition of the host-cell plasma membrane and has been suggested to alter trafficking of the SCV. To examine its role in lysosomal avoidance, we pre-labeled lysosomes with DQ-BSA and then infected cells with either WT or a *sopB* deletion mutant of *S.* Typhimurium. SCVs containing the *sopB* mutant fused with lysosomes more often, as marked by a DQ-BSA signal. In cells transiently transfected with small Rab GTPases fused to GFP, *sopB* mutant bacteria also recruited more GFP-Rab8B, -Rab13, -Rab23, and -Rab35 to the SCV at one hour post infection. Since Rab23 and Rab35 were shown to play a role in phagosome-lysosome fusion, we investigated the mechanism of specific Rab displacement from the SCV by SopB. We found that the prenylated carboxy-terminal polybasic tail of Rab35 localized preferentially to SCVs containing *sopB* mutant rather than WT bacteria, with the same frequency as the full length Rab35, and that both prenylation and polybasic residues were necessary for these membrane interactions. Furthermore, using a fluorescent cationic membrane probe Rpre-Red, we observed that during invasion WT bacteria rapidly loose the negative charge associated with the plasma membrane from the nascent SCVs, while *sopB* mutant bacteria do not. Hence, we propose that through the action of SopB, *S.* Typhimurium can control SCV maturation by altering the SCV membrane charge. This globally affects the recruitment/retention of host-cell protein machinery involved in endocytic trafficking, such as Rab35, and prevents targeting of bacteria in SCVs for lysosomal degradation.

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The Bacterial Virulence Factor InlC Perturbs Apical Cell Junctions and Promotes Cell-cell Spread of Listeria.

T. Rajabian[1], S. Müller-Altrock[2], W. Goebel[2], S. Gray-Owen[1], K. Ireton[3]; [1]Department of Molecular Genetics, University of Toronto, Toronto, ON, Canada, [2]Biocenter (Microbiology), University of Würzburg, Würzburg, Germany, [3]Department of Molecular Biology and Microbiology, College of Medicine, University of Central Florida, Burnett School of Biomedical Sciences, Orlando, FL

Several pathogenic bacteria, including Listeria monocytogenes, use an F-actin dependent motility process to spread between mammalian cells 1. Actin 'comet tails' propel Listeria through the cytoplasm, resulting in bacteria-containing protrusions that are internalized by neighboring cells. Here, we identify bacterial and host proteins that directly regulate the formation of protrusions during spreading. First, we show that efficient cell-cell spread in polarized epithelial cells requires the secreted Listeria virulence protein, InlC. We next identify the mammalian adaptor protein Tuba as a ligand of InlC. InlC binds to a C-terminal SH3 domain in Tuba, which normally engages the human actin regulatory protein N-WASP 2. We show that InlC promotes protrusion formation by inhibiting Tuba and N-WASP function, most likely by impairing binding of N-WASP to the Tuba SH3 domain. Tuba and N-WASP are known to control the structure of apical junctions in epithelial cells 3. We show that, by inhibiting Tuba and N-WASP, InlC transforms taut apical cell-cell junctions into structures with a "slack" morphology. We then demonstrate that this defect in junction structure accounts for the role of InlC in protrusion formation. Collectively, our results suggest that InlC enhances bacterial dissemination by relieving cortical tension in apical junctions. Diminished tension likely enhances the ability of motile bacteria to deform the plasma membrane into protrusions

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A Novel Mechanism of Cytotoxicity by Pseudomonas Aeruginosa and Characterization of RAW 264.7 Cell Lines that Resist Infection.

S. Roy, A. Tartakoff; Department of Pathology and Cell Biology Program, Case Western Reserve University, Cleveland, OH

Pseudomonas aeruginosa is an opportunistic gram-negative pathogen. Since macrophages are significant components of host defense against bacterial pathogens, we have studied the toxicity of *P. aeruginosa* toward the murine monocyte-macrophage cell line, RAW 264.7. Cells exposed to strain PAO1 for 1 hr at MOI 10 did not show immediate evidence of damage or phagocytose bacteria; however, during the following 24-36 hr cells increased dramatically in size, with some becoming multinucleate, and ultimately > 70% died, as judged by MTT assays. Contact between bacteria and host cell, rather than soluble products, is responsible for toxicity. Interestingly, although the *P. aeruginosa* Type III secretion system can secrete toxins directly into the target cells which act on the actin cytoskeleton, rhodamine-phalloidin staining showed that actin filaments of RAW 264.7 are only minimally affected. Consistent with this observation, toxicity requires neither the Type III (nor Type II) secretion systems, judging from experiments with corresponding mutants. To identify molecular targets required for toxicity, RAW 264.7 cells were subjected to ten cycles of exposure to PAO1, each of which kills the large majority of cells. The surviving cells resist exposure to as high as MOI 100 of *P. aeruginosa*.Resistance is a stable characteristic, judging from the behavior of clonal populations. Upon exposure to P. aeruginosa, the resistant clones do not increase in size or become multinucleate. A ConcanavalinA-resistant MDCK mutant is resistant to *P. aeruginosa* (Fleiszig et al., 1997), and this resistance is thought to reflect a requirement for surface

glycans which bind bacteria. We have monitored lectin sensitivity of the cycled RAW264.7 cells which tolerate PAO1 and observe marked resistance to ConA. This linkage between lectin sensitivity and resistance to *P. aeruginosa* could implicate the same surface glycans in both events. Supported by a grant from the Cystic Fibrosis Foundation.

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Vesicle Trafficking as an Intrinsic Cellular Defense Mechanism against Pore-Forming Toxins.

F. Los[1,2], C. Kao[1], R. V. Aroian[1]; [1]Cell & Developmental Biology, University of California, San Diego, La Jolla, CA, [2]Developmental Biology, Utrecht University, Utrecht, Netherlands

The Aroian lab studies the effects of pore forming toxins (PFTs), which form the largest class of proteinaceous bacterial virulence factors. Many well known pathogenic bacteria, such as *Staphylococcus aureus* and *Streptococcus pyogenes*, employ PFTs to facillitate host pathogenesis. A better understanding of how PFTs function and how host cells respond to these toxins will provide new insights into how bacterial pathogenesis occurs, and can prove useful in fighting bacterial infections. Our lab uses *Caenorhabditis elegans* and the *Bacillus thuringiensis* PFT Cry5B as a model, which gives us access to genetic tools to study how target cells respond to and protect against PFTs. Based on studies in mammalian cells, we hypothesized that *C. elegans* cells treated with PFTs would undergo an increase in endocytosis. Using two fluorescent markers for the apical plasma membrane we found that the *C. elegans* intestinal cells indeed react to Cry5B exposure with a rapid upregulation of endocytosis. We identified several vesicle trafficking components that, when eliminated by RNAi, perturb this toxin-induced endocytosis. These same genes were found to lead to hypersensitivity to Cry5B when their functions were disrupted by mutations or RNAi. We conclude from these results that *C. elegans* employs vesicle trafficking and endocytosis in the defense against PFTs. We are currently using a focused RNAi approach to elucidate new components involved in the vesicle trafficking-mediated response to PFTs, as well as genes involved in other defense mechanisms such as the unfolded protein response.

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Two Novel Membrane-Binding Domains of Helicobacter Pylori Protein CagA and Their Function In Vitro.

C. Pelz, C. Weiss, R. Vogelmann; Internal Medicine II, Klinikum rechts der Isar, Munich, Germany

The H. pylori protein CagA is a bacterial oncogene, which is delivered via a type IV secretion system into host cells of the gastric mucosa. In vitro, the known biological effects are assigned to the C-terminus of CagA. Little is know about the role of the remaining two-thirds of this 130 kDa protein in host cells. One report indicates that it contains a membrane-binding domain (Bagnoli et al., PNAS, 2005), which is in contrast to a second report suggesting that the EPIYA motif in the C-terminus is required for membrane targeting (Higashi et al., JBC, 2005). Our goal was to identify the membrane binding domains of CagA in order to determine their role for host cell biology. Using various CagA mutants expressed as transgenes in MDCK cells, we are able to show, that the first 200 AA of CagA are required to localize the protein to the apical and basal-lateral membrane of polarized MDCK cells. In our hands, the C-terminus of CagA (AA 800-1216) containing the EPIYA motifs is predominately found in the cytoplasm with little membrane binding capacity. To our surprise, a CagA mutant missing the first 200 AA (CagA 200-1216) is almost exclusively localized to the apical membrane of polarized MDCK cells leading to its dramatic constriction. Furthermore we can show that the first 200 AA of CagA inhibits the cell elongation phenotype induced by the EPIYA motifs in the C-terminus. Whereas 34% of cells expressing the CagA mutant 800-1216 are elongated in polarized MDCK cells, only 12% of cells transfected with a CagA Δ200-800 mutant reveal this phenotype (18% for wildtype CagA, respectively). We conclude that CagA contains two different membrane-binding domains, one of which inhibits a known CagA effect on host cell biology.

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Protease-Dependent Activation of the Drosophila IMD Pathway in Response to Gram-Negative Bacterial Infection.

R. L. Schmidt[2], F. M. Rinaldo[2], S. E. Hesse[1], M. Hamada[2], A. Page-McCaw[3], J. L. Platt[4], A. H. Tang[1,2]; [1]Surgery, Mayo Clinic College of Medicine, Rochester, MN, [2]Department of Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN, [3]Department of Biology and Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY, [4]Surgery, University of Michigan, Ann Arbor, MI

Gram-negative bacterial infection activates the immune deficiency (IMD) pathway, leading to production of antimicrobial peptides (AMPs), as a part of the Drosophila host defense system. As a receptor for the IMD pathway, peptidoglycan-recognition protein (PGRP), PGRP-LC is thought to recognize and bind monomeric peptidoglycan (DAP-type PGN) through its PGRP ectodomain and in turn activate the IMD pathway. Since abundant "commensal" bacteria are coexisting peacefully in healthy animal hosts without eliciting eminent host immune responses, the questions remain how PGRP-LC differentiates pathogenic versus nonpathogenic Gram-negative bacteria, both with the common pathogen-associated molecular patterns (PAMPs) such as PGN, and how PGRP-LC is activated in response to pathogen infection to initiate the IMD signal transduction in Drosophila. Here we present evidence to show that proteases such as elastase and Mmp2 can activate the IMD pathway but not the TOLL pathway. This elastase-dependent IMD activation requires the receptor PGRP-LC. We find that live Salmonella/E.coli infection modulates PGRP-LC expression/receptor integrity and activates the IMD pathway while dead Salmonella/E.coli or protease-deficient E. coli do neither. Our results suggest a likely possibility that Gram-negative pathogen infection may be partially monitored through the structural integrity of the receptor PGRP-LC via an infection-induced cleavage-mediated activation mechanism.

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Cell Death in HepG2 Cells Infected with a Virulent Klebsiella pneumoniae.

J. Wu; Microbiology and Immunology, Chang Gung University, Tao Yuan, Taiwan

Background: Virulent *Klebasiella pneumoniae* (KP) inflicts severe liver abscess in infected patients although non-virulent KP is considered as normal intestinal flora in some individuals. How the bacteria cause cell death is not clear. **Objective:** To investigate how virulent KP causes liver cell death. **Methods:** A KP strain isolated from a patient with liver abscess and proved to be highly virulent in mice was used to infect HepG2 cells. Western blotting was used to examine various molecules involved in cell death pathway in cell extract harvested at different time points after infection. Infected cells were also investigated for DNA laddering and cell cycle change by flow cytometry.

Results: The infection caused cell arrest at cell cycle G1 phase 6 to 7 hours after infection. Apoptosis changed to secondary necrosis was observed during the early phase of infection. At 9 hours after infection, primary necrosis was greatly increased. DNA laddering was apparent at 8 hours post-infection and lasted even to 16 hours. Small amount of caspase 3 was cleaved 2-6 hours post-infection but not at later time, so was the PARP cleavage. Both Bax and Bcl-2 were decreased at 6-12 hr post-infection. Bcl-XL was significantly decreased 6-10 hr post-infection. **Conclusion:** HepG2 cells infected with a virulent KP are arrested at G1 phase and display apoptosis at early stage of infection, with a gradual increase to secondary necrosis. At late stage of infection, primary necrosis is predominant for cell death. DNA laddering is apparent in all cell death period although necrosis is the primary cause of cell death.

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Role of the Sel1-domain Protein EnhC in Host Cell Entry by the Obligate Intracellular Bacterium Coxiella burnetii.

S. D. Gilk[1], S. L. Cirillo[2], J. D. Cirillo[2], R. A. Heinzen[1]; [1]Coxiella Pathogenesis Section, Rocky Mountain Labs, National Institutes of Health, Hamilton, MT, [2]Department of Microbial and Molecular Pathogenesis, Texas A&M University, College Station, TX

Coxiella burnetii is a gram negative obligate intracellular bacterium and the causative agent of human Q fever. *Coxiella* is unique among bacterial pathogens in that it thrives in the acidic environment of a modified phagolysosome. While displaying *in vivo* tropism for mononuclear phagocytes, *Coxiella* promiscuously infects a wide range of cells *in vitro*, including fibroblasts. Upon host cell contact, receptor-ligand interactions trigger classical actin-dependent phagocytosis. In macrophages, unknown bacterial ligands engage the CR3 and integrin αvβ3 receptors. Receptor-ligand interactions have not been identified in cells other than macrophages. In addition, the *Coxiella* proteins involved in manipulation of host cell phagocytosis have not been described. In the related bacterium *Legionella pneumophila*, the protein EnhC is implicated in pathogen entry into both macrophages and epithelial cells. Like *Legionella* EnhC (LpEnhC), *Coxiella* EnhC (CbEnhC) contains multiple (21) Sel1 repeats that mediate protein-protein interactions. A polyclonal antibody against CbEnhC significantly reduced *Coxiella* uptake by HeLa cells, suggesting a role for CbEnhC in *Coxiella* entry. CbEnhC also complemented the entry defect of a *Legionella* enhC deletion mutant. In an extensive two-hybrid screen, the only interacting protein identified was Merlin, a protein that links the actin cytoskeleton with the plasma membrane. Both *Coxiella* and *Legionella* displayed increased entry into Merlin knockout cells, while entry of another bacterial pathogen, *Chlamydia trachomatis*, was unchanged. The *Coxiella* parasitophorous vacuole in Merlin knockout cells appeared less organized, though bacterial growth was not inhibited. Ongoing experiments are addressing the interaction between CbEnhC and Merlin, and how this interaction might promote bacterial uptake by host cells.

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Macrophage Inhibitory Cytokine 1 (MIC-1) is Modulated by Enteropathogenic E. Coli (EPEC) Infection in Human Intestinal Epithelial Cells.

H. Choi, Y. Moon; Department of Microbiology and Immunology, Pusan National University School of Medicine, Busan, South Korea

Enteropathogenic E. coli is an extracellular bacterial pathogen that infects the human intestinal epithelium and is a major cause of infantile diarrhea in developing countries. EPEC alters many functions of the host intestinal epithelia and triggers cellular death cell progress such as apoptosis and necrotic cell death. Macrophage Inhibitory Cytokine 1 (MIC-1) is originally identified as a regulatory cytokine after inflammation and recently also characterized as a typical member of the TGF-beta super family which modulates cellular growth, survival, apoptosis and differentiation both in the normal and transformed cells. In this study, the involvement of MIC-1 protein in cellular progress in the EPEC-infected epithelial cells was addressed using the co-culture with the human intestinal epithelial cells. EPEC was a potent inducer of MIC-1 expression which was associated with infection-mediated apoptosis. EPEC treatment increased MIC-1 protein and mRNA levels in a time- or dose-dependent manner. When MIC-1 mRNA was blocked by siRNA, EPEC-induced cellular apoptosis was attenuated, which was observed by Annexin-propidium iodide FACS analysis. This was also confirmed by PARP protein degradation. Moreover, in terms of gene expression signals, MIC-1 induction was mediated by GSK3beta activation by EPEC. Conclusively, EPEC induced MIC-1 gene expression in the human intestinal epithelial cells, which contributed to the apoptotic cell death after the pathogenic bacterial attachment in the mucosal surface.

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Recombinant Lectin-like Domain of Thrombomodulin Reduces Klebsiella Pneumoniae-induced Inflammatory Responses.

H. Wu[1,2], C. Shi[1,2], G. Shi[1,2]; [1]Department of Biochemistry and Molecular Biology, National Cheng Kung University, Tainan, Taiwan, [2]Cardiovascular Research Center, National Cheng Kung University, Tainan, Taiwan

Septic shock syndrome resulting from excessive host immune responses induced by infectious organisms is a leading cause of death in hospitalized patients. Gram-negative bacterial infection is one of the major causes of systemic bacterial sepsis. Thrombomodulin (TM), a widely expressing glycoprotein and originally identified in vascular endothelium, is an important cofactor in the protein C anti-coagulant system. Previous reports demonstrated that TM lectin-like domain (TMD1) possesses anti-inflammatory property through protein C independent pathway. This study aims at investigating the effects of recombinant TMD1 (rTMD1) on Gram-negative bacteria-induced inflammatory responses. rTMD1 was prepared and purified using both Pichia pastoris and mammalian protein expression systems. Effects of rTMD1 treatment on the Klebsiella pneumonia (K. pneumonia) -induced inflammatory mediator productions and lethality were studied. In vitro experiment showed that tumor necrosis factor-α and nitric oxide levels were increased within 12 hours in mice receiving K. pneumonia, and rTMD1 treatment effectively reduced the tumor necrosis factor-α and nitric oxide production. In addition, in the in vivo survival experiment, all mice received K. pneumoniae died within 18 hours, whereas 50% of the mice received a single i.v. dose of rTMD1survived more than 24 hours. rTMD1 also significantly enhanced K. pneumoniae clearance in circulation. In conclusion, we presently have demonstrated that rTMD1functions as a protective agent against sepsis caused by Gram-negative bacterial infections.

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Mechanism of Actin Cross-linking with ACD Domain of V. Cholerae MARTX Toxin.

D. Kudryashov[1], E. Chen[1], K. Satchell[2], E. Reisler[1]; [1]Chemistry and Biochemistry, UCLA, Los Angeles, CA, [2]Microbiology and Immunology, Northwestern University, Chicago, IL

Multifunctional Autoprocessive Repeats in Toxins toxin (MARTX) of V. cholerae disrupts actin cytoskeleton by cross-linking actin monomers into arrays of oligomers in a Mg/ATP dependent manner. This activity has been attributed to ~450 a.a. long central part of MARTX named Actin Crosslinking Domain (ACD). Recently we demonstrated that ACD catalyzes a formation of an iso-peptide bond between the Glu270 residue of one actin subunit to the Lys50 residue on another subunit. Due to the spatial constrains, the cross-linked subunits fail to polymerize unless the assembly is supported by phalloidin or cofilin. ACD does not share significant similarity with any known enzyme and the precise mechanism of its activity remains unknown. In this report we continue our exploration of the enzymatic properties of ACD. Upon incubation with gamma P32-ATP, ACD incorporates inorganic phosphate into the K50C but not the E270Q actin mutant. This indicates that the cross-linking reaction proceeds via formation of a Glu270-acyl-phosphate intermediate. In the case of wt actin, the activated Glu270 is then cross-linked efficiently to Lys50 on another actin protomer. Interestingly, when Lys50 is unavailable due to mutation, Glu270 can be attached, although with lower efficiency, to other substrates. By blocking the growth of oligomers at one of the ends, we show that actin subunits are incorporated at similar rates on both ends. Finally, we demonstrate that Mg-GTP can be also used as an energy source for the cross-linking reaction, albeit with lower efficiency than Mg-ATP. This finding may be relevant for in vivo conditions, because GTP is present in eukaryotic cells at high concentrations. The mechanism of actin cross-linking by ACD via an acyl-phosphate intermediate resembles Mur catalyzed amino acid addition to the peptidoglycan during bacterial cell wall synthesis. Yet, a lack of similarity between Mur enzymes and ACD indicates that these proteins evolved independently.

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E. coli CNF1 Toxin: Ubiquitinated-Rac Partners for Efficient Host-Cell Invasion.

L. Boyer[1], O. Visvikis[1], M. Lemonnier[1], A. Doye[1], E. Veiga[2], P. Lores[3], G. Flatau[1], G. Gacon[3], P. Cossart[2], E. Lemichez[1]; [1]INSERM, Nice, France, [2]Microbiology, Pasteur, Paris, France, [3]Genetic, Inst. Cochin, Paris, France

Studies on the interaction of bacterial pathogens with their host have provided an invaluable source of information on the function of key regulators of endocytic and phagocytic processes. Bacterial factors exploit eukaryotic cell machineries using refined molecular strategies to promote invasion and proliferation within their host, as well as modulate innate immune responses. Here, we present new results on CNF1 a bacterial toxin from uropathogenic E. coli. This toxin modulates the activity of Rho GTPases by exploiting the ubiquitin/proteasome machineries. CNF1 triggers a permanent activation of Rho proteins, maximale for Rac, which next leads to the sensitization of Rho proteins to ubiquitination and proteasomal degradation. Both the activation and the ubiquitination of Rac are responsible for a highly efficient invasion of bacteria into non-professional phagocytic epithelial and endothelial cells. Using two hybrid screening preformed with a chimeric Ubiquitin-Rac molecule, we have identified cell factors, which interfere with the induction of bacterial internalization triggered by Rac and its ubiquitination. Our study on CNF1 toxin, the paradigm of Rho GTPase-activating toxins, reveals important connections between bacterial invasion triggered by Rho GTPases and the Toll-like receptor pahway.

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An RNAi Screen Reveals That Host Abi/WAVE And Arp2/3 Complexes are required for Rickettsia Parkeri Invasion.

S. C. Reed[1], M. Welch[1,2]; [1]Microbiology Graduate Group, University of California, Berkeley, Berkeley, CA, [2]Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Many intracellular bacterial pathogens invade host cells by exploiting actin-dependent pathways. Although the molecular mechanisms of actin assembly during invasion have been extensively investigated for some pathogens, little is known about the invasion of *Rickettsiae*, a group of bacterial pathogens that cause spotted fever and epidemic typhus. Previous work has shown that proteins important for *Rickettsia* invasion include actin, tyrosine kinases, the Rho-family GTPase Cdc42, and the Ku70 subunit of DNA dependent protein kinase. To further define the host factors that participate in *Rickettsia* entry, we used an RNAi-based screening approach in *Drosophila* S2R+ cells to determine which of 107 known cytoskeletal-associated host proteins were required for efficient entry of *Rickettsia parkeri*. We found that RNAi silencing of 37 of the 107 gene targets resulted in a statistically significant reduction in the percentage of intracellular bacteria following a 15 min infection. Proteins important for entry included actin nucleators, Rho family GTPases, adapter proteins, kinases, myosins, membrane-cytoskeleton linkers, and actin organizers. The most severe entry defects occurred in cells silenced for the expression of the nucleation promoting factor (NPF) WAVE, members of the WAVE complex, and subunits of the Arp2/3 complex. Surprisingly, RNAi silencing of a related NPF, WASP, did not result in reduced internalization. WAVE was also specifically implicated in *Rickettsia* invasion of mammalian cells, as entry was defective in WAVE2 knockout mouse cells, but not N-WASP knockout cells. Furthermore, RNAi experiments in human cells confirmed that the Arp2/3 complex, the WAVE complex member Abi and the Rho GTPases Cdc42 and Rac1 were important for entry. Overall, these results define an actin nucleation and signaling pathway utilized during *Rickettsia* entry, and will aid in the search for additional host receptors, kinases, and bacterial effectors involved in this process.

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Study on Role and Efficiency of Differential Expression of InlA and InlB in Understanding Listeria Pathogenicity.

Y. Woo, A. Artyukhin, A. Hiddessen, T. Sulchek; BBTD, Lawrence Livermore National Lab, Livermore, CA

Pathogens have evolved to accommodate environments of different host cells. Listeria monocytogenes is a gram-positive bacterium that has two very different lives. It is well adapted as a saprophyte in soil, but it has a second life as a pathogenic bacterium causing serious infection in humans and many animals. How Listeria switches between the two lives may be attributed to differential expression of invasion proteins, InlA and InlB, in response to changing environment. To study this hypothesis, we coated 2 µm polystyrene beads with various amount of InlA and InlB and incubated them with different mammalian epithelial cells. Internalization efficiency of the beads at each internalin concentration was measured by the antibody quenching method using a fluorescence microscope. We found that internalization efficiency for InlA- and InlB- coated beads showed significant difference depending on the host cells and protein concentrations. We also noticed that InlA-coated beads require significantly lower amount of protein for internalization than InlB. This study will contribute to understanding how

Listeria may control its internalization efficiency in different host cell environments and will help to elucidate how InlA and InlB function differently in bacteria's invasion strategy.

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Trafficking of Mycobacterium tuberculosis Bacilli in Alveolar Epithelial Cells.

K. Fine[1], R. Pavlicek[1], E. White[2], M. Metcalf[2], F. Quinn[1]; [1]Infectious Diseases, University of Georgia, Athens, GA, [2]Infectious Disease Pathology, Centers for Disease Control and Prevention, Atlanta, GA

Most pulmonary pathogens associate intimately with alveolar epithelial cells at some stage of the disease process. However, the role these cells play in the pathogenesis of tuberculosis (TB) is not known. It is thought that upon inhalation, *M. tuberculosis* bacilli are rapidly engulfed by alveolar macrophages and dendritic cells. Dissemination from the lungs is believed to occur as infected phagocytes traffic to regional lymph nodes. A *M. tuberculosis* mutant, (ΔnecA), was recently developed and found to produce 50-70% less necrosis than the virulent parent strain, Erdman, when examined on infected A549 alveolar epithelial cells. This mutant also disseminates 50-70% less efficiently from the lungs of aerosol-infected mice. These observations suggest that mycobacterial interaction with alveolar macrophages and dendritic cells may not be sufficient to produce disease pathogenesis and that there may be an additional requirement for alveolar pneumocytes. In addition to ΔnecA and Erdman, two other less-virulent mycobacterial strains also were examined: *M. tuberculosis*, ΔHBHA, a mutant lacking the heparin-binding hemagglutinin surface adhesin, and *M. bovis*, BCG Pasteur (BCG), the live attenuated TB vaccine strain. Using electron and confocal microscopy, we demonstrated that all four strains attach to and are internalized via an actin pseudopod engulfment mechanism. However, only 1% of Erdman bacilli appear to be internalized by 3-4 hr post infection (p.i.) compared to 4-5% for ΔHBHA, BCG and ΔnecA. This ratio decreases to 1:3 by 18 hr p.i. Also, the ΔnecA bacilli seem to transit more rapidly through the vacuolar system with approximately 10-15% of the infecting bacilli escaping from phagosomes and residing in the cytosol by 24 hr p.i. while the virulent parent bacilli remain in vacuoles. Lastly, we observed exocytosis occurring within 2-3 days after infection with the virulent mycobacteria; this observation has not been observed with the mutant strains suggesting a potential cell lysis and dissemination mechanism. Findings from these experiments suggest a role for epithelial cells in pulmonary disease and demonstrate that mycobacterial trafficking in epithelial cells differs from that observed in macrophages.

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Legionella Pneumophila Infections Involve Bacterial F-box Proteins.

M. Lomma[1], D. Dervins-Ravault[1], H. Newton[2], F. Samson[2], M. Jules[1], E. Hartland[2], C. Buchrieser[1]; [1]Unité de Biologie des Bactéries Intracellulaires, Pasteur Institute, Paris, France, [2]Department of Microbiology, Monash University, Victoria, VIC, Australia

Legionella pneumophila is the etiological agent of Legionnaire's disease. This Gram-negative bacterium is present in fresh and artificial water environments and replicates in protozoan hosts. When aerosolized bacteria are inhaled, they can colonize the respiratory tract, invade alveolar macrophages and replicate therein causing the disease. Analysis of the genome sequence of L. pneumophila revealed the presence of an unexpected high number and variety of eukaryotic-like proteins, predicted to be involved in the exploitation of the host cellular cycle by mimicking eukaryotic functions. Among these are three proteins containing F-Box domains. In eukaryotes, F-Box proteins are E3 ubiquitin-ligases that act in the last step of the ubiquitination process by recognising specific substrates. The aim of this study is to determine the role of these proteins in the virulence and their interference with the ubiquitination pathways of the host cells. We thus generated all possible combinations of F-box mutants by replacing the F-box domain of each gene of interest with a different antibiotic resistance cassette. When tested for their capacity to infect and replicate in the infection model Acanthamoeba castellanii, the mutants were clearly impaired in intracellular replication and virulence suggesting that they indeed may be involved in exploiting the eukaryotic ubiquitination machinery. To asses the role of the F-box proteins also in human infection, we are currently testing the mutants in additional infection models like human monocytes THP-1 and lung epithelial cells A549. Furthermore we performed in vivo competitive virulence assays of the mutants and wild-type L. pneumophila strains in a pulmonary mouse model (A/J mice). First results again suggest their implication in replication in vivo and in the different cell lines. A Yeast Two Hybrid screen identified two possible targets for one of the F-box proteins (Lpp2082). Further investigations to validate these targets in in vivo assays, such as pull-down and co-immunoprecipitation, expriments are under way to determine at which stage of the infection they may act and whether they are degraded by the proteasome following ubiquitination.

693/B656

VIP Ameliorates C. Rodentium-induced Disruption of the Colonic Epithelial Barrier.

V. Conlin[1,2,3], X. Wu[1,2], C. Dai[1,2], B. A. Vallance[1,2], A. M. Buchan[3], K. Jacobson[1,2,3]; [1]Child and Family Research Institute, BC Children's Hospital, Vancouver, BC, Canada, [2]Division of Gastroenterology, BC Children's Hospital, Vancouver, BC, Canada, [3]Cellular & Physiological Sciences, UBC, Vancouver, BC, Canada

Background Bacterial infections of the gastrointestinal tract disrupt epithelial barrier integrity, increasing permeability and allowing luminal content access to the underlying milieu. Gastrointestinal epithelium has a high density of neuropeptide receptors and is therefore particularly responsive to their effects. We examined the role of Vasoactive Intestinal Peptide (VIP) treatment to modulate Citrobacter rodentium-induced disruption of intestinal epithelial barrier integrity. Methods Colitis was induced in C57BL/6 mice by oral gavage with C. rodentium. VIP was given daily by subcutaneous injection (0.5, 1, 5 nmol); body weight was assessed daily and mice were euthanized on day 10 post-infection. Epithelial Paracellular Permeability- distal colonic segments were mounted in Ussings chambers and mannitol flux assessed. Immunofluorescence- frozen colonic tissues were labeled with anti-VIP, -ZO-1, -occludin, and -claudin 3. Western blotting- colonic lysates were analysed by SDS-PAGE and membranes probed for anti-MLCK and phospho-MLC. Results VIP had no significant effect on bacterial attachment, but ameliorated colitis-induced epithelial damage. 0.5 nmol VIP treatment proved most effective. Infection caused a 32% [± 2%] decrease in weight at day 4 p.i. ($p<0.001$ vs. control); in contrast VIP attenuated weight loss (16% [± 4%] decrease compared to non-infected mice ($p<0.01$). Infected animals demonstrated significantly lower body weight at day 10 ($p<0.001$), whereas VIP treatment resulted in body weights similar to control (p=NS). VIP attenuated infection-induced increase in permeability (p=NS vs. infected mice, $p<0.01$) and prevented disruption of ZO-1, occludin and claudin-3. In addition, VIP treatment attenuated the 6-fold increase in C. rodentium-induced MLCK

expression (p<0.001) and the 3-fold increase in MLC phosphorylation revealing levels similar to control (p=NS). Conclusion C. rodentium altered colonic epithelial barrier integrity, disrupting tight junction proteins and increasing permeability. Our results indicate that VIP protected colonic epithelial barrier integrity by attenuating redistribution of tight junction proteins in part through actions on MLCK and MLC phosphorylation.

Parasitology (694-702)

694/B657

Characterization of a Novel Plasmodium Falciparum Effector That Interacts With Glycophorin Binding Protein 130 and the Host Cytoskeleton.

G. Mayer; Virginia Commonwealth University, Richmond, VA

After invading a host erythrocyte, P. falciparum induces modification of the host erythrocyte, a cell devoid of organelles and protein trafficking machinery. These modifications allow the parasite access to nutrients and alter the host to provide a more favorable environment for the parasite. We have identified a novel P. falciparum-exported protein lacking an obvious PEXEL motif, PfEffector B, which shares homology to Effector protein B, an L. pneumophila effector of the Dot/Icm system. We have generated antisera against PfEffector B to determine whether PfEffector B is exported to the host erythrocyte. Our data show that PfEffector B is expressed at all stages of the intraerythrocytic cycle and is exported to the host erythrocyte. Moreover, we have shown that PfEffector B colocalizes and interacts with GBP130, a soluble PEXEL-positive P. falciparum-exported protein, and spectrin. This study identifies a novel PEXEL-negative P. falciparum-exported protein which shares homology to effector protein B of L. pneumophila and interacts with GBP 130 and the host cytoskeleton. We propose that the analysis of PfEffector B might add a valuable component to the currently predicted exportome of P. falciparum and shed light on how proteins without an obvious PEXEL are exported into the host cell.

695/B658

Inhibition of Intracellular Proteolysis in Malaria Parasites.

M. L. Gazarini[1], L. Juliano[2], M. A. Juliano[2], C. Garcia[3], R. L. Cunha[4]; [1]Biosciences, UNIFESP, Santos, Brazil, [2]Biophysics, UNIFESP, São Paulo, Brazil, [3]Physiology, USP, São Paulo, Brazil, [4]Exact and Earth Sciences, UNIFESP, Diadema, Brazil

The control of malaria is being challenged by the increasing resistance to available drugs. In this scenario, protease inhibitors emerge as possible therapeutic agents for malaria because they are able to block parasite intracellular development and erythrocyte invasion and evasion, and, in some cases, kill the parasite in vitro through protease inhibition. We developed a drug screening assay using a rodent malaria model with Plasmodium chabaudi where it is possible to follow the intracellular parasite proteolysis as previously described (Farias SL. et al, Mol. Biochem Parasitol.2005). Herein we report our results on the evaluation of the effect of organotelluranes in intracellular proteolysis using Z-FR-AMC substrate. Initially a screening of a set of compounds was performed with a solution containing freshly extracted, intact P. chabaudi trophozoite cells, in both pH 6.5 and 7.0, which are physiologically representative for Plasmodium infection. Thus, RF05 and RF19 compounds were found as the most potent in tested pHs. The determined IC50 values for other compounds, at pH 6.5, were 235nM for the ionic derivative RF03 and 8uM for RF07. Since RF03 is the most hydrophilic member of the set, it was chosen for a preliminary evaluation of its chemotherapy potential. A group of mice were treated for 10 days with RF03. This non-optimized treatment increased mice survival significantly. Our data provided a new approach for studies of protease activity in the cytosol of malaria parasites taking advantage of the promiscuous capacity of substrate internalization of P. chabaudi cells. Our cell-based assay is useful to observe and measure the intracellular proteolysis after cell signaling changes (calcium homeostasis) and its inhibition being useful to find novel potential compounds for malaria treatment. This work is supported by FAPESP and CNPq.

696/B659

Potential Contribution of Autophagy to the Metamorphosis of the Malaria Parasite within the Hepatocyte.

B. Jayabalasingham, I. Coppens; Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD

The malaria parasite undergoes several obligate developmental changes over the course of its life cycle. One such change occurs after invasion of the mammalian host liver, where the *Plasmodium* parasite converts from a motile, elongated sporozoite into a spherical replication competent trophozoite. This metamorphosis is accompanied by dramatic cellular changes. A major component of the sporozoite's cytoskeleton, which is composed of a double membrane cisternae called the inner membrane complex, is packaged and released from the parasite, permitting its sphericalization and expansion. Additionally, micronemes, secretory organelles that facilitate sporozoite invasion are clustered and eliminated. Upon completion of metamorphosis, the parasite retains a minimal set of organelles involved in biosynthesis and replication consisting of the ER, mitochondria, nuclei, and the apicoplast, the site of type II fatty acid synthesis. The mechanisms involved in this metamorphosis remain unknown. Our electron microscopy studies reveal the presence of autophagosome-like structures containing sporozoite organelles at the onset of transformation. In addition, the classical autophagy inhibitor 3-methyladenine inhibits commitment to conversion. We have cloned the malaria homologue of the yeast autophagy related gene, *ATG8* from *Plasmodium berghei*, *Pbatg8* and confirmed that it is highly expressed in both sporozoites and intrahepatic parasites. Pbatg8 shares 67% similarity with its yeast homologue and is localized to tiny vesicle-like structures dispersed in the parasite cytoplasm. By yeast 2 hybrid, we have demonstrated that Pbatg8 interacts with a *P.berghei* homologue of the autophagy related protein, Atg3. We are currently in the process of assaying the functional homology of these *Plasmodium* autophagy related genes by confirming conjugation of Pbatg8 to phosphatidylethanolamine, and complementing *Δatg8 S. cerevisiae* with Pbatg8. Together, our results support the involvement of autophagy in the early stages of intracellular replication of *Plasmodium* in liver cells. These advances lay the groundwork for dissection of the developmental pathways implicated in the dramatic metamorphic changes of the malaria parasite.

697/B660

Dissection of Intermediate Filaments and MORN1 in Toxoplasma gondii Cell Division.

B. Anderson-White, N. Sahoo, K. Yang, A. Lorestani, K. Cheng, S. Robertson, M. Gubbels; Biology, Boston College, Chestnut Hill, MA

The apicomplexan parasite *Toxoplasma gondii* divides asexually through a unique process of internal daughter budding that functions by elusive mechanisms as yet only visually defined. Through the study of key elements in cytoskeleton assembly, we aim to further define division and cytoskeleton formation. We have focused on two proteins that dynamically associate with the cytoskeleton during budding: TgMORN1 and TgIMC3. TgMORN1 is one of the first identifiable components of the forming daughter scaffold and forms an integral component of the spindle pole and the apical and basal complexes. In addition, TgMORN1 localizes to the elongating inner membrane complex (IMC; a cytoskeleton structure comprised of intermediate filaments and vesicular sacs) during division, suggesting that TgMORN1 may function in IMC organization. Our co-immunoprecipitation data shows that TgMORN1 complexes with TgIMC1, an intermediate filament protein, and supports this assertion. We are currently investigating the role of TgMORN1 in division and cytoskeletal development by identifying members of the TgMORN1 complex through fractionation and yeast two hybrid method to reveal the proteins associated with TgMORN1. A second line of work pertains the association of TgIMC3, another intermediate filament protein, with the budding daughter cytoskeleton. Initial results suggest TgIMC3 is strongly cell cycle regulated and is modified throughout endodyogeny. This suggests TgIMC3 plays a role in cytoskeleton assembly and we plan to define the function of TgIMC3 in this capacity. Furthermore we initiated a comprehensive study of the intermediate protein family in *T. gondii*. We anticipate these studies will highlight mechanisms that control the parasite-specific components of the division machinery.

698/B661

Characterization of the Endocytic Pathway and Use as an Iron Source of Ferritin by Entamoeba Histolytica.

L. F. López[1], C. Piña[1], N. Leon-Sicairos[2], A. Gonzalez-Robles[3], M. de la Garza[1]; [1]Cell Biology, Cinvestav-IPN, México D.F., Mexico, [2]Medicine, University Autonomic of Sinaloa, Culiacan, Mexico, [3]infectomic and Molecular Pathogenesis, Cinvestav-IPN, Mexico

Iron is an essential element for growth of nearly all organisms; it is not free into the cell due to its toxicity. Ferritin is an iron-storage protein containing up to 4500 atoms of Fe (III). Ferritin is mainly present in liver, spleen and brain in mammals. Reports about using host ferritin as iron source by pathogens are a few. Several studies have been done regarding ferritin endocytosis, for instance, in oligodendrocytes, ferritin was internalized via clathrin-coated vesicles. Previous work of our group has shown that Entamoeba histolytica, the parasite causing amoebiasis, utilizes human hemoglobin, transferrin and lactoferrin as iron sources for growing. In liver, amoeba could interact with ferritin, which probably provides enough iron for the parasite survival. We searched whether E. histolytica trophozoites utilize ferritin as an iron source for growth in axenic cultures; we also characterized the endocytic pathway by confocal and electronic microscopy, flow cytometry, and substrate gel electrophoresis. Amoebas preserved their viability (80%) in 50 µg/ml ferritin (100 µM iron). Cysteine proteases from amoebic crude extracts cleaved ferritin producing several fragments of different molecular weight. Amoebas endocytosed FITC-ferritin and cationized-ferritin, process initiated at 1-2 min of incubation; after this time, ferritin was located inside of vesicles in cytosol. Ferritin endocytosis was through clathrin vesicles, since it was inhibited with chloroquine and other classical clathrin inhibitors; this was evaluated by flow citometry, confocal microscopy and transmission electron microscopy. Also, we found by overlay assay that amoeba has a ferritin binding protein of 140 kDa. The ferritin endocytosis was dependent of its binding-protein, because this route was inhibited by trypsin treatment on surface. Ferritin endocytosis behaved as a regulated process leading to saturation of the ferritin binding-protein. The ferritin binding protein was specific to ferritin, since hemoglobin and transferrin did not block the binding of ferritin. The capacity of amoebas to use ferritin for surviving could help explain the parasite virulence in liver and the cause of hepatic abscess.

699/B662

Characterization of Autophagy Protein 8 Mediating Autophagosome in Encysting Acanthamoeba.

E. Moon, D. Chung, Y. Hong, H. Kong; Kyungpook National University School of Medicine, Daegu, South Korea

Autophagy is an evolutionally conserved protein degradation pathway in eukaryotes. It plays essential roles during starvation, cellular differentiation, cell death, and aging by eliminating unwanted or unnecessary organelles and recycling the components for reuse. ATG8, a member of a novel ubiquitin-like protein family, is an essential component of the autophagic machinery. In the present study, we identified and characterized an autophagy protein 8 in encysting Acanthemoba castellanii (AcAtg8). Atg8 of A. castellanii encodes 118 amino acids and shown to be highly expressed during encystation by real-time PCR. The pUb vector expressing EGFP-Atg8 fusion protein was transfected to analyze the intracellular localization of Atg8 during encystation. Fluorescence microscopic analyses showed small or large vacuolar fluorescent structures in encysting amoeba. Fluoresence microscopy revealed the structures with Atg8 on the membrane were autophagosomes by co-localization analysis with Lysotracker. Chemically synthesized small interfering RNA (siRNA) against Atg8 reduced the encystation efficiency and inhibited the formation of autophagosome during encystation. Identification of Atg8 from encysting Acanthamoeba would open the way for further study on autophagosome mechanism of cyst-forming pathogenic protozoa.

700/B663

The Unusual 10-Formyl-THF Metabolism of Leishmania Major Is a Drug Target.

T. J. Vickers[1], S. M. Murta[1], D. A. Scott[2], S. M. Beverley[1]; [1]Molecular Microbiology, Washington University, St Louis, MO, [2]Burnham Institute, La Jolla, CA

Leishmania are protozoan parasites that infect humans. These organisms are folate auxotrophs that show many unusual aspects in their pterin metabolism, which makes these metabolic pathways attractive drug targets. In other organisms, 10-formyl-tetrahydrofolate (10-formyl-THF) is used in *de novo* purine biosynthesis and the formylation of the mitochondrial initiator tRNA. In mammals, 10-formyl-THF is synthesized in the cytoplasm by the trifunctional enzyme methylenetetrahydrofolate dehydrogenase-cyclohydrolase/formyl-tetrahydrofolate ligase (DHCH-FTL) called the C1 complex, or in the mitochondrion by a bifunctional DHCH. However *Leishmania major* instead has single separate DHCH and FTL genes and, since it is a purine auxotroph, the parasite is predicted to only require 10-formyl-THF in the mitochondrion. Unexpectedly, sub-cellular fractionation showed that the *L. major* DHCH and FTL proteins were not found in the

mitochondrion and were instead cytoplasmic. Characterization of recombinant DHCH and FTL showed these proteins had the expected enzymatic activities, and that DHCH could be potently inhibited by a transition state analogue. This inhibitor was toxic to the parasite. Null mutants of *L. major* lacking FTL were viable and had normal growth, differentiation and virulence in animal infections. In contrast, although DHCH heterozygotes were readily obtained, attempts to generate dhch- null mutants yielded only aneuploids, bearing both planned replacements but retaining a copy of DHCH gene. This indicates that DHCH is an essential gene. Chromosomal null mutants could be obtained following introduction of an episomal DHCH or FTL. Negative selection using thymidyate kinase or GFP/FACS sorting against an episomal copy of DHCH failed to recover viable null mutants, despite analysis of >1000 events. These data argue that 10-formyl-THF is an essential metabolite that is produced in the cytoplasm and consumed in the mitochondrion. This highly unusual form of folate metabolism has considerable potential as a drug target.

701/B664

Toll-like Receptor 4 Knock-out Mice Display Persistent C. Parvum Biliary Tract Infection and Cholangiocyte Proliferation.

S. O'Hara[1], P. S. Bogert[1], C. E. Trussoni[1], A. J. Stroope[1], J. Lewis[2], N. F. LaRusso[1]; [1]Center for Basic Research in Digestive Diseases, Mayo Clinic, Rochester, Rochester, MN, [2]Anatomic Pathology, Mayo Clinic, Rochester, MN

Upon pathogen recognition, Toll-like receptors (TLRs) initiate host defense responses to mobilize immune effector cells and activate adaptive immunity. Using an in vitro model of biliary cryptosporidiosis we previously showed that Cryptosporidium parvum, a parasite that infects both intestinal and biliary epithelia, induces a TLR4-dependent antimicrobial response in cholangiocytes (epithelial cells lining the bile ducts). Disruption of the TLR4 signal pathway in cultured cholangiocytes resulted in a reduced capacity to clear C. parvum infection. Here we tested the role of TLR4 during C. parvum biliary infection using an in vivo model of biliary cryptosporidiosis in both wild type (WT) and TLR4 knock-out mice. Mice were infected via direct injection of C. parvum (200,000 oocysts) into the gallbladder and sacrificed at 1,2,3 and 4 weeks post infection; control animals received injections of saline. Serum chemistries, liver histology, parasite clearance, cytokine production, and cholangiocyte proliferation were assessed. WT mice displayed a transient biliary tract infection peaking at 2 weeks post-infection as determined by histology and microscopic examination of stool and bile. Serum ALT and AST, TNF-α, interferon-γ, IL-6 and the antimicrobial peptide, beta-defensin, peaked at one to two weeks post-infection and normalized by four weeks. Proliferating cell nuclear antigen (PCNA) staining was elevated at one-week post-infection but was not detected thereafter. In contrast, the TLR4 knock-out mice displayed persistent biliary tract infection with elevated serum ALT and AST through four weeks post-infection. Histology remained abnormal and cholangiocyte proliferation persisted through four weeks post-infection. Interestingly, beta-defensin was not elevated in the knock-out mice, while IL-6 levels were elevated throughout the time-course. These data suggest that a TLR4-mediated response is required for in vivo eradication of biliary C. parvum infection and the lack of this pattern recognition receptor contributes to proliferation and an altered inflammatory response.

702/B665

Motile Behavior and Chemosensory Responses in the Parasitic Flagellate Trichomonas vaginalis.

M. Levandowsky; [1]Haskins Labs, Pace University, New York, NY, [2]Humanities and Sciences, School of Visual Arts, New York, NY

Swimming behavior and sensory responses of the flagellate, Trichomonas vaginalis were studied. Using videomicroscopy we determined swimming speed and traced swimming paths of cells in TYM medium with and without 10% added serum. In media containing serum or dialyzed serum the cells form dynamic aggregates. Oxygen gradients did not affect this behavior. Swimming speed for cells not involved in the aggregates was the same as in media without serum. We measured path-length and mean square displacement for large numbers of cells over different time intervals. Dspersion followed a power law, suggesting the possibility of a Levy distribution, with power law slope of 1.35. Chemosensory responses were measured using a system of chambers with putative chemical cues,connected by small apertures, allowing cells to swim from one to another, but with almost no mixing of solutions between chambers. Cells preferentially aggregated in chambers containing putrescine 1mM.

Cancer I: Signaling (702A-730)

702A/B667

p120 a Critical Nodal Point for HER2/Neu-Induced Breast Cancer Cell Behaviors.

E. Johnson, C. M. De León Rodríguez, D. Seachrist, R. A. Keri; Pharmacology, Case Western Reserve University, Cleveland, OH

HER2/Neu is a member of the Epidermal Growth Factor Receptor family. Its overexpression occurs in 20-30% of all breast cancers, and is associated with poor clinical outcome and survival. Elucidating the underlying mechanisms governing the formation and metastatic progression of HER2-positive disease should identify novel therapeutic targets. Gene expression profiling of the MMTV-c-Neu mouse model of HER2/Neu positive breast cancer revealed a 2.5 fold increase in p120ctn mRNA expression. Analysis of public array data also indicates a high correlation (R=0.806) between HER2/Neu and p120 mRNA in various normal tissues. p120 is known to modulate many cellular activities including cell-cell adhesion and migration. Given its multifaceted role, we hypothesized that p120 functions as an integral signaling node for HER2/Neu-induced breast cancer. Cell lines derived from MMTV-c-Neu mouse mammary tumors (EN) display increased p120 and E-cadherin levels compared to normal mammary epithelium. Furthermore, the overexpression of Neu in human mammary epithelial cell line MCF-10A (10AN) and heregulin stimulation of SkBr3 cells results in elevated p120 protein levels. The use of pharmacological inhibitors revealed that p120 expression is dependent on Akt and Src activity. shRNA attenuation of p120 expression in EN and 10AN cell lines severely abrogates Neu-induced wound healing, cell migration and invasion. Neu-induced activation of RhoGTPases is also suppressed upon p120 knockdown. In addition, p120 knock-down diminishes cell proliferation in response to HER2/Neu. These results are further corroborated in 3-D matrigel cultures and BT-474 xenografts where p120 silencing inhibits colony or tumor growth, respectively. To determine if the Neu-induced increase in p120 expression was sufficient to convey Neu-induced cell behaviors, p120 was overexpressed in

MCF-10A cells. p120 overexpression increased migration/invasion, cell proliferation, branching morphogenesis and RhoGTPase activity. These data indicate that p120 plays a pivotal role in mediating HER2/Neu-induced tumor cell behavior.

703/B668

Cell-based Delivery of Decorin Blocks the Proliferation of Mouse Mammary Tumor Cells.

A. Gingery[1], A. Greene[3], J. Holy[2], T. Rose-Hellekant[1], E. L. Perkins[3]; [1]Medical School - Dept of Physiology and Pharmacology, University of Minnesota, Duluth, MN, [2]Medical School - Dept of Pathology & Laboratory Medicine, University of Minnesota, Duluth, MN, [3]Dept of Basic Medical Sciences, Mercer University School of Medicine, Savannah, GA

Decorin, a leucine rich proteoglycan, regulates collagen fibrillogenesis, cell growth and extracellular signaling. Extracellular signaling by decorin occurs through the binding and inhibition of TGF-β's and through activation of EGFR. Decorin suppresses growth of many tumor cells through binding and activation of EGFR/ErbB1, which ultimately arrests cells in the G1 phase of the cell cycle. Decorin protein levels are low in the micro-environment of many transformed cells. Enforced expression of decorin, and increased decorin protein levels in cancer cells suppresses tumor growth and metastasis. Here we tested whether in vitro, cell-based production of decorin would be an effective delivery vehicle in order to arrest tumor cell growth. Our data demonstrates that delivery of decorin from CHO cells is effective in inhibiting mammary tumor cell proliferation through the EGFR signaling and regulation. In parallel, we are optimizing delivery of decorin from mesenchymal stem cells (MSCs) to test whether MSC produced decorin exhibits antitumor properties. Given that MSCs home to tumor sites, we propose that MSCs may be an ideal decorin delivery vehicle for cell-based antitumor factor production. In this study we test the ability of MSCs, transfected with decorin, to inhibit growth of isogenic tumor cells, as a model for autologous, cell-based anti-tumor therapy.

704/B669

The Ras-like Tumor Suppressor DiRas3 (Noey2, ARHI) Downregulates Specifically the RAF-MEK-ERK Signaling Pathway by Inhibiting MEK.

M. Klingauf[1], M. Beck[1], A. Robubi[2], M. Hekmann[3], A. Baljuls[3], S. Heinzer[1], Y. Turgay[1], U. R. Rapp[3], R. Kroschewski[1]; [1]Institute of Biochemistry, ETH Zuerich, Zuerich, Switzerland, [2]Department of Bioinformatics, University of Wuerzburg, Wuerzburg, Germany, [3]Institute for Medical Radiation and Cell Research, University of Wuerzburg, Wuerzburg, Germany

The Ras-RAF-MEK-ERK pathway is hyper-regulated in about 30% of solid tumors. Ras-like DiRas3 (ARHI, Noey2) is encoded by a maternally imprinted gene, often lost in breast and ovarian cancer and described as tumor suppressor. Previously it was shown that its reexpression in cancer cells suppressed ERK phosphorylation and proliferation. However, the molecular mechanisms by which DiRas3 exerts these functions are not known. We demonstrate that DiRas3 is N- and C-terminally tethered to the plasma membrane. It acts specifically in the mitogenic signaling pathway through inhibition of MEK. DiRas3 binds also to active C-RAF, however, it did not inhibit its kinase activity towards MEK *in vitro*. These data suggest, that DiRas3 utilizes membrane localized active C-RAF as a linker to MEK. ERK phosphorylation sustained longer at a higher level in stimulated knockdown cells which mimic cancer cells. This reveals an inhibitory buffer function of DiRas3 on the Ras-RAF-MEK-ERK pathway in normal tissue. As the nucleotide binding state of DiRas3 did not influence its association to C-RAF, we propose that RAF-MEK-ERK signaling might be modulated at the level of DiRas3 expression.

705/B670

RSK2 Controls Cell Survival through Spatial Restriction of the Apoptosis Promoting Factor, TIA-1, to Stress Granules.

T. Eisinger-Mathason[1,2], D. E. Clark[1], L. Pasic[1], T. L. Muratore-Schroeder[3], J. Shabanowitz[3], D. F. Hunt[3], I. Macara[1,2], D. A. Lannigan[1,2]; [1]Center for Cell Signaling, University of Virginia, Charlottesville, VA, [2]Department of Microbiology, University of Virginia, Charlottesville, VA, [3]Department of Chemistry, University of Virginia, Charlottesville, VA

RSK2 is a Ser/Thr kinase downstream of ERK1/2. Until recently, our understanding of RSK2 function has been limited to its role in cell growth and development. We have found a novel, unanticipated link between stress granules and RSK2. RSK2 is a key component of stress granules in MCF-7 cells subjected to oxidative stress (sodium arsensite, 500 μM; 1hr). Stress granules are RNA granules that form in the cytoplasm of cells exposed to various types of stress. Specific mRNAs are stored in the granules and are protected from decay. These mRNAs are available for immediate translation when the stress abates. Both specific knockdown of RSK2 and treatment with the RSK-specific inhibitor, SL0101 (100 μM; 4h), reduce stress granule formation by 75% and 50% respectively in response to oxidative stress. These findings suggest that RSK2 kinase activity is essential for stress granule formation. We have found, by GST-binding assay, that recombinant RSK2 interacts directly with the essential pro-apoptotic stress granule marker, TIA-1. Using RSK2 and TIA-1 deletion mutants we have shown that the N-terminal portion of RSK2, amino acids 1-389, interacts with the C-terminal prion-like domain of TIA-1. Consistent with these findings, we have observed that an N-terminal mutant RSK2, RSK2(1-389), is able to localize to stress granules upon oxidative stress. However, the C-terminal mutant RSK2(373-741) cannot localize to stress granules. These data suggest that the N-terminal domain of RSK2 regulates stress granule formation via its interaction with TIA-1. Additionally, a RSK2 kinase dead mutant, RSK2(K100A), can neither bind TIA-1 nor localize to stress granules. These data are consistent with the observation that inhibition of RSK2 kinase activity decreases stress granule formation. Importantly, decreased RSK2 levels significantly reduced the ability of breast cell lines to survive oxidative stress by increasing apoptosis. Consistent with these data, knockdown of RSK2, by short hairpin RNA, in primary human epithelial cells followed by treatment with sodium arsenite caused an increase in cleaved caspase 3 levels. Together, these data indicate that RSK2 functions in cell survival by facilitating stress granule formation.

706/B671

Mitogen Limitation or Inhibition of ERK Activation Prevent Apoptosis Induced by Nitric Oxide Donor, Nitroprusside.

L. Gomez-Sarosi, M. Strasberg-Rieber; Tumor Cell Biology, CMBC, IVIC, Caracas, Venezuela

Nitric oxide (NO) is capable of promoting death or survival depending on cell type and experimental conditions. NO has been linked to tumor progression by promoting angiogenesis, and invasion. On the other hand, NO can mediate the tumoricidal effect of macrophages (Wink, D. et al, Nitric Oxide,19:65 ,2008) and be a survival factor for normal lens epithelial cells (Chamberlain, C.G. et al, Mol Vis.

;14:983,2008) .NO donors also can affect conventional therapy. The nitric oxide donor, sodium nitroprusside (SNP) is used as a hypotensive agent during clinical anaesthesia, and to improve hemodynamic index in advanced heart failure (Chalon,S. et al. Br. J. Clin. Pharmacol.47:91,1999). OBJECTIVE. Since ERK activation is important in mitogenesis but also may be critical for survival in a stressful NO microenvironment , we now investigated the B16 melanoma response to nitroprusside under mitogen-restrictive or permissive conditions, in the presence or absence of UO126, a specific pharmacological inhibitor of ERK activation . METHODS. cell viability was assessed by Alamar Blue fluorescence; survival was determined by DNA content; chromatin condensation and DNA damage estimated by the TUNEL assay were quantitated by laser scanning cytometry (Strasberg-Rieber et al, Apoptosis, 12 : 449,2007). RESULTS. B16 melanoma treatment with nitroprusside in medium supplemented with 10 % serum led to loss of survival . This response was not seen after 3 days of serum depletion in cells treated with nitroprusside , in which loss of DNA content did not occur compared to serum depleted cells without nitroprusside. . Serum supplementation greatly augmented apoptosis-associated chromatin condensation and TUNEL reactivity, in cells treated with nitroprusside Both nitroprusside -mediated survival with serum depletion or its pro-apoptotic effect with serum-supplementation , were antagonized by inhibition of ERK activation . CONCLUSIONS. Although NO donors exert paradoxical concentration-dependent effects ,this report shows that a fixed concentration of nitroprusside promotes survival or apoptosis depending on mitogen availability or extent of ERK activation in tumor cells .

707/B672

Activation of Akt by DNA-dsbs and its Possible Role in DNA Double-Strand Break Repair and Cell Survival.

M. Fraser[1,3], F. Jalali[1,3], R. Bristow[1,2,3]; [1]Cellular Imaging Core, STTARR Innovation Facility/ Radiation Medicine Program/Princess Margaret Hospital, Toronto, ON, Canada, [2]Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada, [3]Applied Molecular Oncology, Ontario Cancer Institute/Princess Margaret Hospital, Toronto, ON, Canada

Activation of the serine/threonine protein kinase Akt is commonly observed in numerous human cancers and associated with tumour progression and resistance to DNA damaging agents. Recent evidence suggests that activated Akt is required for effective repair of double-strand DNA breaks (DSB). However, very little is currently known about the mechanism(s) by which Akt can facilitate DNA repair and cell survival in the context of genetic instability and therapy resistance. We are currently investigating the dose- and time-dependent regulation of Akt phosphorylation and subcellular localization induced by DNA-dsbs (ionizing radiation; IR) and the role of phosphorylated Akt as a key mediator of cell survival following DNA-dsbs. Using western blotting and immunofluorescence microscopic analyses, we observed that IR induces phosphorylation of Akt on S473 (P-Akt) in DU145 and 22RV1 prostate cancer cells with increased focal nuclear accumulation of P-Akt. The DNA damage-associated formation of P-Akt foci was not associated with a change in sub-nuclear localization of total Akt. P-Akt co-localized with the DNA-dsb biomarker gamma-H2AX following both whole-cell and sub-nuclear UV microbeam-induced DNA-dsbs in ATM- and DNA-PKcs-deficient cells, suggesting that P-Akt foci are independent of these two DNA-dsb repair kinases. Akt1 siRNA sensitized cells to IR. Additionally, chemical inhibition of Akt signaling lowered P-Akt levels and inhibited Akt, 53BP1 and BRCA1 DNA repair foci in a MDC1-independent manner. Akt inhibition led to increased residual gamma-H2AX foci, suggesting that Akt may play a role in the repair of IR-induced DSB. Taken together, these data suggest that Akt phosphorylation is induced by IR, possibly through an ATM and DNA-PKcs-independent mechanism, and that P-Akt is a determinant of the survival response to IR.

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PTEN and PHLPP1 Tumor Suppressors are Recruited to the Membrane by NHERF1 to Synergistically Inhibit Akt.

J. R. Molina, F. Morales, Y. Hayashi, M. Georgescu; Neuro-Oncology, UT MD Anderson Cancer Center, Houston, TX

Glioblastoma is a frequent brain malignancy with poor prognosis. PTEN tumor suppressor antagonizes PI3K and is frequently deleted in glioblastoma. We unexpectedly found that most glioblastoma cells expressing endogenous PTEN have basal PI3K/Akt activation, caused especially by impaired PTEN plasma membrane distribution. This alteration coincided with aberrant cytoplasmic shift of NHERF1, which normally contributes to PTEN membrane recruitment. In cells exhibiting membrane NHERF1, only simultaneous PTEN and NHERF1 depletion activated Akt, implying additional PI3K/Akt suppressors regulated by NHERF1, identified here as the PHLPP1-2 Akt phosphatases. NHERF1 directly interacted and recruited PHLPP1-2 to the membrane. Simultaneous elimination of PTEN and PHLPP was necessary and sufficient to upregulate Akt in cells with membrane-localized NHERF1 and synergistically boosted cancer cell growth. Analysis of glioblastoma lines and tumors showed frequent NHERF1 membrane loss. Conversely, NHERF1 membrane targeting suppressed Akt and cell proliferation. Our findings reveal a novel mechanism for PI3K/Akt activation in tumors by synergistic inactivation of PTEN and PHLPP following loss of membrane NHERF1.

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Phosphoproteomic Analysis of Syk Kinase Signaling in Human Cancer Cells Reveals its Role in Cell-Cell Adhesion.

R. M. Larive[1], S. Urbach[2], J. Poncet[2], P. Jouin[2], G. Mascré[1], A. Sahuquet[1], P. H. Mangeat[1], P. J. Coopman[1], N. Bettache[1]; [1]Centre de Recherche de Biochimie Macromoléculaire, CNRS, Montpellier, France, [2]Institut de Genomique Fonctionnelle, CNRS-INSERM, Montpellier, France

The spleen tyrosine kinase Syk has predominantly been studied in hematopoietic cells in which it is involved in immunoreceptor-mediated signaling. Recently, Syk expression was evidenced in numerous non-hematopoietic cells and shown to be involved in tumor formation and progression. Loss of Syk expression in breast tumors correlates with an increased risk for metastasis formation and Syk transfection reduced the tumorigenic and metastatic capacities in mice. The Syk downstream signaling effectors in non-hematopoietic cells remain, however, to be uncovered and were investigated using MS-based quantitative phosphoproteomics. Two strategies, based on the inhibition of the Syk catalytic activity with the pharmacological inhibitor piceatannol in MCF7 breast cancer cells and on the loss of Syk expression in the metastatic MDA-MB-231 and the doxorubicin-resistant MCF7/ADR cell lines were employed to identify phosphotyrosine-dependent complexes. Quantitative measurements were obtained on 350 proteins purified with phosphotyrosine affinity columns using the SILAC (stable isotope labeling with amino acids in cell culture) method. Forty-one proteins are dependent on the Syk expression and catalytic activity and were selected as signaling effectors. They are involved in a variety of biological processes such as signal transduction, cell-cell

adhesion and cell polarization. We investigated the functional involvement of Syk in cell-cell adhesion and demonstrated the phosphorylation of E-cadherin and á-catenin in vitro. In addition, Syk is localized at cell-cell contacts and its transfection induces E-cadherin tyrosine phosphorylation in MCF7 cells. Identification of the biochemical pathways regulated by Syk in human cancer cells will help to uncover its role in tumor formation and progression.

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EGF stimulated Pak1 nuclear import requires a functional interaction with LC8.

C. M. Lightcap[1], L. E. Arias-Romero[2], G. Kari[3], S. Sun[4], T. Polenova[4], J. Chernoff[2], U. Rodeck[5], J. C. Williams[6,1]; [1]Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA, [2]Tumor Cell Biology Program, Fox Chase Cancer Center, Philadelphia, PA, [3]Radiation Oncology, Thomas Jefferson University, Philadelphia, PA, [4]Chemistry and Biochemistry, University of Delaware, Newark, DE, [5]Dermatology, Thomas Jefferson University, Philadelphia, PA, [6]Molecular Medicine, Beckman Research Institute, Duarte, CA

Pak1 (p21-activated kinase-1) and the dynein light chain, LC8, are both overexpressed in breast cancer, and their direct interaction has been proposed to regulate tumor cell survival. These effects have been attributed in part to Pak1-mediated phosphorylation of LC8 at serine 88. However, LC8 is homodimeric, which renders Ser88 inaccessible within the dimeric interface. Moreover, Pak1 does not contain a canonical LC8 binding sequence as compared with other characterized LC8 binding sequences. Together, these observations raise the question whether the Pak1/LC8 interaction is distinct (i.e. enabled by a unique interface independent of LC8 dimerization). Herein, we present results from biochemical and biophysical studies that show that Pak1 (residues 212-222) binds to LC8 along the same groove as canonical LC8 interaction partners (e.g. nNOS and BimL). However, we differentiate Pak1 from canonical LC8 binding partners and identify a key hydrogen bond network that compensates for the loss of the conserved glutamine in the consensus sequence. We also show that LC8 dimerization is required for the LC8/Pak1 interaction to occur and precludes phosphorylation of LC8 at Ser88. Consistent with this observation, in vitro phosphorylation assays using activated Pak1 did not result in LC8 phosphorylation. Instead, we demonstrate that LC8 directs Pak1 nuclear import in MCF7 cells upon EGF stimulation. We also show that this function of LC8 is distinct from its role in dynein-mediated cellular phenomena. Taken together, these results suggest that LC8 directs nuclear import of Pak1 and that nuclear translocation of Pak1 may support breast cancer cell survival.

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Identification and Functional Analysis of Tyrosine Phosphorylation Sites of Abi-1 by c-Abl.

M. Sato, M. Maruoka, T. Shishido; Graduate school of biological science, Nara Institute of Science and Technology, Nara, Japan

The Abelson protein tyrosine kinase (c-Abl) is a non-receptor tyrosine kinase. c-Abl kinase can be intramolecularly folded, resulting in suppression of its activity. Our group has suggested that trans-acting adaptor molecules, such as c-Crk and Abl interactor 1 (Abi-1), are important for the regulation of c-Abl kinase. Previous studies have revealed that the c-Crk adaptor proteins are trans-acting activators of c-Abl kinase and that activation of c-Abl by c-Crk consists of steps that depend on the phosphorylation status of c-Crk. Another adaptor protein, Abelson interactor 1 (Abi-1) protein was isolated as v-Abl kinase binding proteins. Abi-1 promotes c-Abl-mediated phosphorylation of Mammalian Enabled (Mena) and WAVE2. Since the Crk adaptor protein regulates c-Abl kinase through tyrosine phosphorylation of Crk itself, the phosphorylation of Abi-1 may also be involved in the regulation of the c-Abl-mediated phosphorylation of the substrates. Therefore, we tried to identify the phosphorylation sites of Abi-1. Mass spectroscopy analysis revealed that tyrosine 213 and 398 of Abi-1 can be phosphorylated by c-Abl. Mutational analysis showed that these two sites were major phosphorylation sites of Abi-1 by c-Abl. Next, we examined the phosphorylation of Abi-1 is relevant to c-Abl-mediated phosphorylation of Mena and WAVE2 by overexpression system in 293T cells. While phosphorylation of WAVE2 was unchanged, tyrosine mutant form of Abi-1 led to decreased phosphorylation of Mena, as compared with wild type Abi-1. These results indicate that phosphorylated form of Abi-1 is involved in the recognition of substrate by c-Abl.

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VASP is an Abi-1-Regulated Substate of Abl Kinases.

M. Maruoka, M. Sato, T. Shishido; Biological Science, Nara Institute of Science and Technology, Ikoma, Nara, Japan

Objective c-Abl is a proto-oncogene of v-Abl tyrosine kinase which is encoded in Abelson murine leukemia virus (A-MuLV). Although oncogenic form of v-Abl and BCR-Abl has unregulated kinase activities, the kinase activity of c-Abl is tightly regulated, and activation mechanism of c-Abl is not well understood. Previously, our group demonstrated that Abi-1 binds to both c-Abl and the substrate, Mammalian enabled (Mena), and promotes phosphorylation of the substrates by Abl kinases. Recently, Mass spectrometric large-scale analysis reported that Vasodilator stimulated phosphoprotein (VASP) is tyrosine phosphorylated in BCR-Abl transformed leukemic cell lines. VASP is a family of Mena and is implicated in cytoskeleton reorganization, cell adhesion, motility, and integrin-mediated “inside-out” signaling. From these reports, we thought that VASP could serves as an Abi-1-mediated substrate of Abl kinases. Methods and results By using overexpresion system, we confirmed that Abi-1 promotes tyrosine phosphorylation of VASP by c-Abl and BCR-Abl. To identify the phosphorylation sites of VASP by Abl kinases, We constructed mutant forms of VASP which substituted tyrosine with phenylalanines. These plasmids were expressed with Abi-1 and c-Abl or BCR-Abl, and analysed by western blotting. As a result, tyrosine at position 39 of VASP is phosphorylated by Abl kinases. Next, we analysed cell adhesion to extra-cellular matrix in BCR-Abl-transformed Ba/F3 cells. Expression of Wild-type VASP is not significantly affected the adhesion to Fibronectin in BCR-Abl-transformed Ba/F3 cells. However, expression of phospho-tyrosine mutant VASP, tyrosine 39 substituted with phenylalanine, decreased the adhesion to Fibronectin. Conclusion From these results, we identified VASP as an abi-1-regulated substrate of Abl kinases. Expression of tyrosine mutant form of VASP decreased adhesion to fibronectin in BCR-Abl-transformed Ba/F3 cells, indicates that phosphorylation of VASP is important for cell adhesion to fibronectin. These results may lead to one explanation of BCR-Abl induced invasion of leukemic cell transformation.

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Inhibition of K-RAS-Mediated Tumorigenesis and Metastasis by Blocking SIAH E3 Ligase-Dependent Proteolysis in Pancreatic Cancer.

R. L. Schmidt[2], C. H. Park[1], A. U. Ahmed[1], N. R. Reed[1], J. H. Gundelach[1], B. E. Knudsen[1], A. H. Tang[1,2]; [1]Surgery, Mayo Clinic College of Medicine, Rochester, MN, [2]Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN

Pancreatic cancer is the most lethal form of human cancers known; the median survival of pancreatic cancer is only 6 months and the mortality rate is 95%. As such, there is an urgent need to understand the molecular events leading to the genesis of pancreatic cancer, and to identify new and effective therapy directed at these molecular alterations in order to successfully treat this lethal disease. The central importance of the K-RAS pathway activation has been well established in pancreatic cancer. Oncogenic K-RAS mutations are found in virtually all pancreatic adenocarcinomas and it is the major signaling pathway that is known to drive aggressive cellular transformation, oncogenesis and metastasis in pancreatic cancers. Thus, an important goal in pancreatic cancer biology is to identify means of countervailing activated RAS signals and reverse malignant transformation. We found that the human homologue of a highly conserved family of Drosophila RING domain E3 ubiquitin ligases - SEVEN-IN-ABSENTIA (SINA), SIAH was specifically and markedly upregulated in pancreatic cancers. The increased SIAH expression seemed to correlate with increased grades and aggressiveness of pancreatic cancer. SIAH was required for mammalian K-RAS signal transduction. We demonstrated that by inhibiting SIAH function, we were able to completely abolish both TUMORIGENESIS and METASTASIS of human pancreatic cancer cells in athymic nude mice. These results suggest that SIAH may be an attractive new target for novel anti-RAS and anti-cancer therapy in pancreatic cancer. Thus, developing SIAH-based anti-cancer agents may offer a new way to inhibit K-RAS-mediated tumorigenesis and metastasis in pancreatic cancer. Since the substrate specificity of ubiquitin-mediated proteolysis is primarily determined by the E3 ligases, our results have provided new insights into the altered proteolysis in the genesis of human pancreatic cancer, and may lead to new avenues for pancreatic cancer diagnosis and novel SIAH-based anti-RAS and anti-cancer therapy that could lead to efficacious treatments that could halt the progression and metastasis of human pancreatic cancer and save the lives of millions of individuals with this often fatal diseases.

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Ron-Dependent Beta-Catenin Activation is Associated with Human and Murine Breast Tumorigenesis.

P. Wagh, S. Waltz; Cancer and Cell Biology, University of Cincinnati, Cincinnati, OH

The Ron receptor tyrosine kinase, a member of the Met receptor family, is overexpressed in variety of human cancers including breast cancer. To delineate the role of Ron in breast tumorigenesis, our laboratory generated transgenic mice that overexpressed Ron selectively in the mammary epithelium. These transgenic mice developed mammary tumors with 100% penetrance, short latency and exhibited lung and liver metastases. Microarray analysis comparing tumors from Ron overexpressing mammary glands and wild type mammary glands identified beta-catenin as one of the genes upregulated following Ron overexpression. Mammary tumors from these mice also demonstrated an elevation in total and phosphorylated beta-catenin. Analysis of human breast cancer specimens and cell lines demonstrated elevated levels of both Ron and beta-catenin coordinately. Further, treatment of Ron overexpressing breast cancer cell lines with an inhibitor of beta-catenin lead to decreased cell viability. Based on this data, we propose to test the hypothesis that beta-catenin nuclear translocation and activation are critical downstream signaling events during Ron-induced mammary tumorigenesis. To test this hypothesis, we show that ligand (hepatocyte growth factor like protein/HGFL)-induced Ron activation leads to the nuclear localization of beta-catenin which is tyrosine phosphorylated. To investigate the tyrosine residues in beta-catenin in which tyrosine phosphorylation may play an important role downstream of Ron activation, analyses of different tyrosine mutants of beta-catenin are being studied. Our preliminary data suggests that tyrosines Y654 and Y670 in beta-catenin are important, as mutation of these residues abolishes beta-catenin nuclear localization following Ron activation. Co-immunoprecipitation studies have also demonstrated that Ron physically associates with beta-catenin and that Ron associated beta-catenin is also tyrosine phosphorylated. In total, these studies suggest that beta-catenin may be a critical mediator of Ron-induced mammary tumorigenesis. Future studies will examine the significance of Ron-dependent beta-catenin tyrosine phosphorylation and nuclear localization in specific aspects of breast tumorigenesis.

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Identification of the Hrb (HIV-1 Rev Binding) Protein as a Target of Oncogenic Notch Signaling.

S. S. Khwaja[1], J. van Deursen[2], R. J. Bram[2]; [1]Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN, [2]Department of Pediatric and Adolescent Medicine, Mayo Clinic, Rochester, MN

A pathophysiologic increase in Notch signaling has been implicated as a causal or contributing factor in the development of T-cell acute lymphoblastic leukemias/lymphomas (T-ALL). Our experiments have identified Hrb (HIV-1 Rev Binding) as an important downstream target of oncogenic Notch signaling. In preliminary experiments, we observed an increase in Hrb transcript and protein levels in T cell lymphomas isolated from mice with conditional deletion of CBP (Creb Binding Protein). CBP-null mice lacking the Hrb gene demonstrated a reduced rate of lymphomagenesis. In addition to an upregulation of Hrb, CBP-null lymphomas also upregulate several Notch pathway components. CBP-null mice crossed to mice with activated Notch developed lymphomas earlier. Previously published reports have suggested that Hrb is regulated by Notch signaling. A close analysis of the Hrb promoter region reveals a consensus CSL binding site, suggesting that Hrb is a direct target of Notch1. This link between Hrb and Notch1 has focused our efforts in understanding the role of Hrb in Notch signaling. To help us understand the role of Hrb in oncogenic Notch signaling, we have adopted the OP9-DL1 co-culture system to support growth of immature thymocytes. Thymocytes transduced with activated Notch1 (ICN1) upregulate Hrb at the protein level. Transduction of thymocytes with activated Notch results in a clear increase in the numbers of immature CD8 single positives (ISP) and CD4/CD8 double positive (DP) cells. Thymocytes lacking Hrb have an altered response to activated Notch signaling. We observed a reduction in the ISP and DP thymocyte numbers. To understand the role of Hrb in ICN1-induced leukemia development, we have utilized a well-characterized bone marrow transplant model. Mice transplanted with ICN1-transduced Hrb -/- bone marrow precursors have a significant delay in the development of leukemia. These data reveal that the development of T-ALL in two different mouse models is greatly reduced when Hrb is lacking and lead us to believe that Hrb provides a signal that is selectively required for Notch-dependent malignant transformation of T cell precursors.

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Investigating the Mechanisms of the Cortical Localization of APC2.

M. Zhou, F. Zhouzheng, B. McCartney; Biological Sciences, Carnegie Mellon University, Pittsburgh, PA

The product of the human tumor suppressor gene *Adenomatous Polyposis Coli (APC)* functions to negatively regulate Wnt signal transduction and to organize the microtubule and actin cytoskeletons. APC proteins reside in various locations within the cell including microtubule plus ends, the cell cortex and the nucleus. These pools of APC likely carry out different cellular functions. We have shown that loss of the cortical localization of Drosophila APC2 correlates with loss of function, leading to mis-regulation of Wnt signaling and disruption of some actin structures. The molecular mechanisms which affect the cortical localization of APC proteins are not well understood. We have demonstrated that in Drosophila Schneider 2 (S2) cultured cells, both the N-terminal and the C-terminal halves of APC2 are required for its proper cortical localization. Further dissection of the C-terminal half of the protein revealed that while the central 20 amino acid repeats are dispensable for APC2's cortical localization, the C-terminal SAMP repeats are required. As a complement to these studies, we are using RNAi to knock down candidate proteins in S2 cells that may play a role in the cortical localization of APC2. Finally, these studies revealed that APC2 may form higher order complexes through a 27 amino acid domain known as conserved region B (CrB). We are currently assessing the function of CrB in the Drosophila embryo.

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Using Site-Directed Mutants of Drosophila APC2 to Investigate the Role of the APC2-Armadillo Interaction in Wnt Signal Transduction and Cytoskeletal Function.

M. Zhou, F. Zhouzheng, B. McCartney; Biological Sciences, Carnegie Mellon University, Pittsburgh, PA

Adenomatous Polyposis Coli (APC) proteins function to negatively regulate Wnt signal transduction and to organize the microtubule and actin cytoskeletons. It is well established that the interaction between the 20 amino acid repeats (20Rs) of APC and β-catenin, a key effector of Wnt signaling, plays an important role in APC's ability to negatively regulate Wnt signal transduction. In addition, some reports suggest that the interaction between APC and β-catenin may also affect cytoskeletal function. Recent models suggest that in the Wnt pathway, APC recruits β-catenin to a destruction complex composed of APC, Axin, GSK3β, and many other proteins, leading to the phosphorylation of β-catenin by GSK3β, and its ultimate ubiquitination, and degradation by the proteosome. Phosphorylation of the 20Rs dramatically increases APC's binding to β-catenin *in vitro*, and recent biochemistry and crystallography data point to key residues in the 20Rs that are important for APC-β-catenin interactions. Based on these data, we generated point mutations of APC2 that either abolish or mimic the phosphorylation at some key residues, or prevent salt bridge formation at others, in some or all of the five 20Rs in APC2. In S2 cells, these mutants localize to the cell cortex like wild type, and they can all be co-immunoprecipitated with Armadillo (fly β-catenin). We are currently testing the *in vivo* functions of these mutant forms of APC2 in the Drosophila embryo. Here we will test specific hypotheses regarding the role of phosphorylation in the APC2-Arm complex and its effect on Wnt signaling and cytoskeletal organization in the early embryo.

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Arachidonic Acid Differentially Modulates the Expression of Gqα, Giα and Gβ in MCF-7 Breast Cancer Cells.

D. Roy, S. Ray, S. Roychowdhury, S. Das; University of texas at el paso, El Paso, TX

Increased synthesis of prostaglandins (PGs) and other arachidonic acid (AA) metabolites is known to induce the growth and proliferation of cancer cells. It is also known that AA and its precursor linoleic acid can be metabolized to produce inflammatory molecules via phospholipase-A2 (PLA2) and cycloxygenase-2 (PLA2/COX-2) reaction pathways. Interestingly, recent results suggest that AA metabolism in breast cancer cells is linked to activation of G-protein coupled receptors (GPCRs). Therefore, we investigated whether G-protein signaling and PLA2/COX-2 pathways are inter-linked and work in tandem in transducing downstream signaling. Using qRT-PCR and immunoblot experiments, we show here that AA activation in MCF-7 cells down-regulates the expression of Gqα and Gβ transcripts by 8 fold but does not alter the expression of Giα. Arachidonyl trifluoromethylketone (ATF), an inhibitor of cytosolic calcium dependant PLA2 (cPLA2), reverses the effect of AA-induced changes in Gβ transcript levels, while having no effect on those of Gqα. However, aristolochic acid that preferentially inhibits secreted PLA2 (sPLA2) down-regulates the expression of Gqα (~9 fold) and Giα (~7 fold) but not Gβ, which indicates that cPLA2 and sPLA2 participate differentially in this process. It is of interest that when MCF-7 cells were treated with G protein agonists, isoproteronol and UK14503, an up-regulation (~50%) of COX-2 and cPLA2 was noted at the protein level, further signifying that hyper AA metabolism through PLA2/COX-2 in MCF7 cells is linked to G-protein signaling. Supported by grants (SO6GM00812 and 5G112RR08124) from the National Institutes of Health.

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Analysis of the Mechanisms that Regulate the Proteasome Inhibition-Induced Nuclear IκBα Translocation in Human Cutaneous T-Cell Lymphoma.

A. Juvekar, H. Vu, C. Ghosh, S. Ramaswami, K. Soe, I. Vancurova; Biology, St Johns University, Queens, NY

The strong resistance to apoptosis in cutaneous T cell lymphoma (CTCL) has been attributed to the high activity of the transcription factor NFκB. Previous studies from our laboratory have shown that the proteasome inhibitor MG132 induces nuclear translocation of IκBα, and this is associated with the inhibition of NFκB activity and increased apoptosis in the CTCL leukemia Hut78 cells. The goal of this study was to use si RNA transfection to determine whether the nuclear IκBα is directly responsible for the inhibition of NFκB activity and increased apoptosis in the leukemia Hut78 cells. In addition, we analyzed whether the MG132-induced nuclear IκBα translocation is reversible in these cells. Our results demonstrate that a decreased nuclear IκBα expression results in an increased NFκB activity, and in the reduction of apoptosis in Hut78 cells. We further show that the nuclear IκBα translocation, induced by proteasome inhibition is irreversible, indicating that the nuclear IκBα is binding to intra-nuclear structures or components. A better understanding of the mechanisms by which the nuclear IκBα regulates the NFκB transcriptional activity in leukemia and cancer cells will contribute to the development of novel anti-cancer therapies. NIH grant GM079581 to I.V.

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Role of IκBα Phosphorylation and Cytoplasmic Concentration in the Proteasome Inhibition-Induced Nuclear IκBα Translocation in the Metastatic Prostate Cancer PC-3 Cells.

H. Vu, S. Ramaswami, C. Ghosh, A. Juvekar, H. Le, M. Hachil, I. Vancurova; Biological Sciences, St.Johns University, New York, NY

We have recently shown that proteasome inhibitors induce the nuclear translocation of IκBα, resulting in the inhibition of NFκB activity and induction of apoptosis in the metastatic prostate cancer PC-3 cells. The goal of this study was to investigate the mechanisms responsible for the proteasome inhibition-induced nuclear IκBα translocation in PC-3 cells. Our results show that phosphorylation of IκBα on Ser 32/36 by IκB kinase (IKK) is not required for the MG132-induced nuclear IκBα translocation in the prostate cancer PC-3 cells. MG132 suppresses the high constitutive activity of the proteasome 26S complex in PC-3 cells, resulting in an increased cytoplasmic IκBα level. However, inhibition of IKK activity, which also causes the increased cytoplamic IκBα level, does not induce IκBα nuclear translocation, suggesting that the increased cytoplasmic accumulation of IκBα is not sufficient to induce the IκBα nuclear translocation, and indicating a specific involvement of the proteasome in the nuclear IκBα translocation. In addition, our data demonstrate that MG132-induced nuclear IκBα interacts with nuclear p65 NFκB, thus providing one of the mechanisms by which the nuclear IκBα inhibits NFκB activity. Since NFκB activity is constitutively increased in many human cancers as well as in inflammatory disorders, the proteasome inhibition-induced nuclear translocation of IκBα could thus provide a new therapeutic strategy aimed at the specific inhibition of NFκB activity by the nuclear IκBα. Funded by: NIH grant GM079581 to I.V.

721/B686

Ubiquitination of HIPK2 by Ubiquitin Ligase SIAH1 Affects Cell Growth and Apoptosis.

S. Kim, D. Choi, C. Choi; Life Science, Sungkyunkwan University, Suwon, Gyeonggi-do, South Korea

Homeodomain-interacting protein kinases (HIPKs) are a family of nuclear serine/threonine protein kinases. The tumor suppressor HIPK2 is an important regulator in a various cellular mechanism, and already known maintained at a low level via ubiquitin-mediated proteolysis. But how its activity is regulated remains largely unclear. In a yeast two-hybrid screen, we identified SIAH1 as a novel interacting partner of HIPK2. SIAH (seven in absentia homolog) is a member of the RING finger E3 ligases and was shown to affect degradation of Tramtrack, a transcriptional repressor of the R7 cell fate during Drosophila eye development. SIAH1 physically interacted with HIPK2 and poly-ubiquitinated HIPK2 in vitro and in vivo. Domain analysis of HIPK2 revealed that ID domain for interaction with transcription factors is responsible for interaction with SIAH1 and C-terminus including ID domain was target for SIAH1-mediated degradation of HIPK2. Degradation of HIPK2 by SIAH1 was blocked and HIPK2 stability was recovered in DNA damage conditions of UV irradiation and Adriamycin treatment. These findings effectively illustrate the regulatory mechanisms by which HIPK2 is maintained at a low level, by SIAH in cells under normal conditions, and stabilized by genotoxic stresses. Our findings suggest that SIAH1 mediated HIPK2 ubiquitination may play a critical role in cell growth and apoptosis in unstressed and damaged cells.

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Stabilization of HIF-1α through Interaction between Src Kinase with PHD2.

H. Lee, H. Park; Life Science, University of Seoul, Seoul, South Korea

Hypoxia-inducible factor (HIF)-1αβ is a heterodimeric transcription factor maintaining O2 homeostasis by inducing angiogenesis, erythropoiesis, and glycolysis. Prolyl hydroxylase domain 2 (PHD2) catalyzes hydroxylation of the proline 564 and /or 402 residues of HIF-1α subunit by using a molecular oxygen. The hydroxylated HIF-1α is ubiquintinated by an ubiquitin E3 ligase, von Hippel Lindau(VHL), then degraded by 26S proteasome. Active Src has been known to accumulate HIF-1α protein by increasing its translation through Akt/mTOR pathway even in normoxia, Here, we investigate whether active Src regulates PHD2 activity. Our finding that Src specific inhibitor, PP2 and tyrosine kinase inhibitor, Genistein increase the hydroxylation-dependent VHL recruitment suggests that Src inhibits PHD2 activity to stabilize HIF-1α. By using GST-pull down assay and co-immunoprecipitation assay, we confirmed that PHD2 directly interacts with Src through its N-terminal region (60-100aa). N-terminal deleted PHD2 mutant but not wild type PHD2 loses its ability to respond to Src, thereby fails to stabilize HIF-1α, suggesting that interaction between Src and PHD2 is important for Src to stabilize HIF-1α. However, our finding that recombinant Src protein fails to prevent purified PHD2 from hydroxylating HIF-1α peptide (556-575aa) implies that Src inhibits PHD2 activity in cellular context, neither by direct interaction nor by phosphorylation of PHD2. In addition, our findings that Src induces reactive oxygen species (ROS) generation, and that DPI, NADPH oxidase inhibitor prevents Src from stabilizing HIF-1α, suggest that through interaction, Src relocates PHD2 around Src associated complexes including NADPH oxidases. Therefore, Src exposes PHD2 to more ROS which inhibits hydroxylation activity. [This work was supported by a grant (R200706192003) from KOSEF. H-Y L is supported by BK21 program].

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Regulation of Groucho Corepressor Activity by Its Covalent Conjugation of SUMO-1.

J. Ahn, Y. Lee; SungKyunKwan Univ., Su-won, Gyeonggi-do, South Korea

Groucho is a corepressor for specific transcription factors which are participated in several development processes including neurogenesis and sex determination. Groucho recruits corepressor complex for its corepressor activity, in which HDAC1 is essential component for transcriptional repression of target genes. Here, we show that Groucho is covalently conjugated by small ubiquitin-related modifier-1 (SUMO-1) in vitro and in vivo. SUMO conjugations of Grocho occur at four different sites which are located in GP and SP domains of Groucho. Substitutions of all lysine residues for sumoylation sites abolished sumoylation of Groucho and inhibited its corepressor activity. In addition, Groucho corepressor activity was reduced by inhibition of SUMO-1 conjugation via Ubc9 knockdown through expression of shRNA against Ubc9. Furthermore, interactions between Groucho and HDAC1, a component of Groucho corepressor complex, are enhanced by sumoylation of Groucho. HDAC1 SIM (Sumo-Interaction Motif) mutant was not recruited to Groucho corepressor complex. Taken together, Groucho sumoylation increases its corepressor activity by enhancing recruitment of HDAC1 to corepressor complex.

724/B689

The Vitamin D Receptor, Hedgehog Signaling and Epidermal Carcinogenesis.

A. E. Teichert[1], J. Welsh[2], D. Bikle[1]; [1]Endocrine Unit, UCSF, San Francisco, CA, [2]Biological Sciences, University of Notre Dame, Notre Dame, IN

Over 1 million skin cancers occur annually in the United States making it the most common cancer. Epidemiologic data support the concept that the incidence of a number of epithelial malignancies is reduced by increasing 25OHD levels. 1,25(OH)2D3, produced by the kidney from 25OHD or locally, reduces proliferation and enhances differentiation and thus has been investigated for a role in preventing or treating cancer. However, UVB, which increases 1,25(OH)2D3 production in the keratinocyte, causes skin cancer. Recent studies show that mice lacking the vitamin D receptor are predisposed to develop such cancers following UVB exposure. Conceivably, the 1,25(OH)2D3 produced by the skin provides at least partial protection from UVB induced skin cancer such that lack of 1,25(OH)2D3 or its receptor would make the skin more sensitive to UVB induced malignancy. We have chosen UVB epidermal carcinogenesis as a model to address the issue of the balance between the risk of UVB exposure as an inducer of cancer with the chemopreventive benefit of promoting vitamin D and thus 1,25(OH)2D3 production in the epidermis. Mice deficient for the vitamin D receptor (VDR null), for which 1,25(OH)2D3 is a ligand, have a marked hyperproliferation in the hair follicle and epidermis and decreased epidermal differentiation. These mice, when treated with UVB, unlike their wildtype littermates, have thickened epidermis with increased hyperproliferation as well as a lower rate of repairing DNA damages such as CpD. To determine the mechanism by which VDR null mice are predisposed to skin cancer, we evaluated the hedgehog (HH) signaling pathway, as disruption of this pathway has been implicated in epidermal carcinogenesis. We found that the epidermis and hair follicles of the VDR null animals, overexpress elements of the HH signaling pathway [Sonic Hedgehog (Shh), Patched 1 (Ptch1), Smoothened (Smoh), Gli 1, and Gli 2]. We also found that the basal expression of these elements and their overexpression in VDR null animals is localized to precise areas of the epidermis and/or hair follicle. These results suggest that the vitamin D signaling mechanism in skin protects it from UVB induced tumor formation by suppression of the HH signaling pathway.

725/B690

RET/PTCs Avoid Lysosomal Degradation to Overcome Low Transcript Copy Numbers and Initiate Papillary Thyroid Carcinoma.

D. S. Richardson, S. Peng, L. M. Mulligan; Pathology and Molecular Medicine, Division of Cancer Biology and Genetics, Queen's University, Kingston, ON, Canada

Chromosomal rearrangements involving the RET gene have been identified in approximately 30 percent of papillary thyroid carcinomas (PTCs), the most common form of thyroid cancer. Although the mechanism by which these rearrangements produce constitutive activation of the RET kinase is well understood, the molecular events that contribute to PTC downstream of RET activation, and the factors that produce variable clinical outcomes depending on the fusion gene involved, have yet to be fully elucidated. Here, we report the opposing roles that decreased transcript levels and altered sub-cellular localization play in the two most common RET rearrangements found in RET/PTCs, PTC1 and PTC3. We show that RET/PTC rearrangements alter sub-cellular localization allowing these proteins to escape lysosomal degradation, increasing activation of the ERK/MAP-kinase pathway. Conversely, transcriptional regulation of the fusion partner genes involved in RET/PTC rearrangements appears to lead to fewer transcripts of these genes, compared to wild type RET. Further, these transcript levels correlate directly with the aggressiveness of PTC1 and PTC3 tumors. Our findings detail a novel mechanism by which RET avoids receptor-mediated endocytosis and the importance of this pathway in the normal downregulation of activated RET. We further suggest that transcript copy number can play a critical role in the expression of fusion proteins and, itself, be a predictor of clinical outcomes in diseases resulting from chromosomal rearrangements.

726/B691

Adenosine Transporter ENT4 Is a Direct Target of EWS/WT1 Translocation Product and Is Highly Expressed in Desmoplastic Small Round Cell Tumor.

H. Li, S. Lee; NIH/NIDDK, Bethesda, MD

Desmoplastic Small Round Cell Tumor (DSRCT) is a highly aggressive malignancy that affects mainly adolescents and young adults. A defining characteristic of DSRCT is a specific chromosomal translocation, t(11;22)(p13;q12), that fuses EWS with WT1, leading to a production of two isoforms of chimeric transcription factor, EWS/WT1(−KTS) and EWS/WT1(+KTS). The chimeric proteins are thought to play critical roles in various stages of oncogenesis through aberrant transcription of different genes, but only a few of these genes have been identified. We report the identification of a new target of EWS/WT1, ENT4 (equilibrative nucleoside transporter 4) which encodes a pH-dependent adenosine transporter. ENT4 is transcriptionally activated by both isoforms of EWS/WT1 as evidenced by promoter-reporter and chromatin immunoprecipitation (ChIP) analyses. Furthermore, ENT4 is highly and specifically expressed in primary tumors of DSRCT as well as in a DSRCT cell line, JN-DSRCT-1. Treatment of JN-DSRCT-1 cells with adenosine analogs, such as 2-chloro-2′-deoxyadenosine (2-CdA), resulted in an increased cytotoxic response in dose- and pH-dependent manner. Our detailed analyses of a novel target of EWS/WT1 in DSRCT reveal an insight into the oncogenic mechanism of EWS-fusion chromosomal translocation gene products and provide a new marker for DSRCT. Furthermore, identification of ENT4 as a highly expressed transcript in DSRCT may represent an attractive pathway for targeting chemotherapeutic drugs into DSRCT.

727/B692

Mutation in DNA-binding Domain of the EWS-Oct-4 Oncogene Results in Dominant Negative Activity That Interferes with EWS-Oct-4 Mediated Transactivation.

S. Kim[1], J. Kim[1], J. Lee[1], E. Shin[1], Y. Han[2], J. Kim[1]; [1]Life Science, Sogang University, Seoul, South Korea, [2]Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, South Korea

The EWS-Oct-4 protein is a chimeric molecule in which the amino terminal domain (NTD) of the EWS becomes fused to the carboxy terminal domain (CTD) of the Oct-4 transcription factor. It was identified in human bone and soft tissue tumors associated with t(6;22)(p21;q12). Using in vitro and in vivo systems, we found that the EWS-Oct-4 protein self-associates. The major domains required for

self-association mapped to the EWS NTD and the POU DNA-binding domain. EWS-Oct-4 protein also associated with EWS-Oct-4 (V351P), wich contains a mutation in the POU DNA-binding domain. Using EMSA, we found that the EWS-Oct-4 (V351P) mutant interfered with wild-type EWS-Oct-4 DNA binding activity. In addition, we found that EWS-Oct-4 mediated transcriptional activation was inhibited by EWS-Oct-4 (V351P) protien in vivo. Thus, this mutation in the POU DNA-binding domain results in a dominant negative protein.

728/B693

Identification of the HspA8 Gene/HSC70 in Regulating Methotrexate Resistance in the L1210 Murine Leukemia Cell Line.

C. K. Daniels[1,3], A. Saini[1], T. Liu[1], P. P. Sheridan[2,3], J. Lai[1,3], A. Bhushan[1,3]; [1]Biomedical and Pharmaceutical Sciences, Idaho State University, Pocatello, ID, [2]Biological Sciences, Idaho State University, Pocatello, ID, [3]The Biomedical Research Institute, Idaho State University, Pocatello, ID

Bhushan and co-workers have previously described an alteration in an unidentified 66-68 kDa membrane-associated, tyrosine-phosphorylated, methotrexate (MTX)-binding protein in the L1210 murine leukemia cell line. This protein was absent, at least in the tyrosine-phosphorylated form, in cisplatin-resistant, methotrexate cross-resistant L1210/DDP cells. Indeed, this 66-68 kDa protein was also demonstrated to be absent, again at least in the tyrosine-phosphorylated form, in cells that were concomitantly rendered MTX-resistant by treatment with the tyrosine kinase inhibitor, genistein. This recently identified 66-68 kDa protein, thus, appeared to be a new, novel player in the mechanisms that have been described accounting for resistance to MTX. We have further characterized this 66-68 kDa protein by affinity chromatography purification using its two identified properties, MTX-binding and tyrosine phosphorylation, yielding a single band of 66-68 kDa on phosphotyrosine western blot. Trypsin digestion and peptide fragments isolation and sequencing yielding two partial peptide sequences: TVTNAVVTVPAYFNDSQRQA and VEIIANDQ. The peptide sequences were used to search the mouse genome at the NCBI database for ORFs containing theses peptides using the TBLASTN function. A single gene was identified containing both sequences, the HSPa8 gene, which codes for the heat shock family protein, HSC70. HSPa8, determined by reverse transcription PCR of mRNA, was found to be transcribed in both cell lines. Western blot analysis showed that HSC70 protein is present in both cell lines. Finally, using a binding assay with MTX-agarose, followed by Western blotting for HSC70, we show HSC70 was present and was found in the MTX-binding fraction. Thus, we have identified a new, novel role for the heat shock protein, HSC70, where an apparent disruption of the normal function of HSC70 can lead to the development of resistance to MTX-induced cell death. This undocumented mechanism may yield an understanding into why some tumor types are unresponsive to MTX treatment, and may provide a new clinical screening tool to determine potential tumor sensitivity to MTX.

729/B694

MGMT Polymorphisms Are Associated with Breast Cancer in Korean Population.

K. Kim[1], S. Lee[1], A. Oh[1], J. Pyun[1], K. Lee[1], H. Song[1], M. Kim[1], S. Won[1], S. Yang[2], W. Han[3], K. Kwack[1]; [1]CHA Research Institute, Pochon CHA Univ., Seongnam, South Korea, [2]Macrogen Inc., Seoul, South Korea, [3]Dept. of Surgery, Seoul National Univ., Seoul, South Korea

O(6)-methylguanine DNA methyl-transferase (MGMT) is the only known critical gene involved in cellular defense against alkylating agents in the DNA repair pathway. We performed genetic association studies in a LD-based breast cancer case-control study analysing polymorphisms in MGMT. The polymorphic variants Leu84Phe and Lys178Arg in MGMT are frequent in the human population. We review here studies of these and other MGMT polymorphisms and their association with risk for breast cancer with a consideration of SNP-hormone receptors (estrogen and progesterone receptor) interactions. Genotyping was performed by GoldenGate assay and included 346 breast cancer patients and 361 normal controls. The frequencies and distributions of alleles, haplotypes, and diplotypes were compared between case and control subjects, as well as between subgroups of case via logistic regression analysis using SAS. A P-value of less than 0.05 was considered to be statistically significant. Forty-nine single nucleotide polymorphisms in MGMT were genotyped in Korean population and multi-marker predictors of common alleles were selected to capture the haplotype patterns specific to each group. We found significant associations between 11 SNPs in MGMT gene and breast cancer patients. Overall, 18 SNPs more significant associated with normal controls and hormone receptor negative (ER(+), PR(-) or ER(-), PR(+)) in breast cancer patients. But, no significant associations was found for C250T (Leu84Phe) and A533G (Lys178Arg) polymorphisms of MGMT in this studied.

730/B695

Role of Peroxisome Proliferator Activated Receptors in Renal Cell Carcinoma.

M. Taub, E. Zaki; Biochemistry, State University of New York at Buffalo, Buffalo, NY

The Renal Clear Cell Carcinoma (RCC), the tenth most prevalent cancer, arises as a result of mutations in the von Hippel-Lindau (VHL) suppressor gene. One consequence of mutations in VHL is the up-regulation of Peroxisome Proliferator-Activated Receptor γ (PPAR γ), that presumably affects PPAR γ mediated signaling events and growth. The objective of this study is to determine the role of PPARs normal growth control and to evaluate the manner in which growth regulation is altered as a consequence of VHL inactivating mutations. Thus, normal renal proximal tubule cells have been examined with regards to their growth responses to PPAR agonists (as the RCC originates from the renal proximal tubule). The primary cultures were initiated from purified rabbit renal proximal tubules in serum free medium supplemented with 5 µg/ ml bovine insulin, 5 µg/ml human transferrin and 5 x 10-8 M hydrocortisone. To examine the role of two classes of PPARs, both fibrate drugs and thiazolidine drugs were examined with regards to their growth stimulatory effects on primary proximal tubule cells. Fibrate drugs and thiazolidinediones (TZDs) have a high affinity for PPAR α and PPAR γ, respectively. The TZD drug troglitazone was examined over a concentration range of 10-7 to 10-4 M. After 7 days cells were detached from culture dishes using trypsin, and counted in a Coulter Counter. The results of 3 independent studies indicate that troglitazone (10-6 to 5 x 10-6 M) increases growth 4-fold. An endogenous PPAR γ, Prostaglandin J2 (PGJ2), was also growth stimulatory. In the kidney both PPAR α and PPAR γ are expressed in the renal proximal tubule. However, fenofibrate, a PPAR α agonist, had no significant affect on renal proximal tubule cell growth over a concentration range of 10-7-10-4 M. Unlike the results that we have obtained with our normal renal proximal tubule cells, recent studies have indicated that troglitazone, and other TZDs (PPAR γ agonists) induce growth arrest and apoptosis in RCC, as well as other urological cancers. We are

currently examining whether inactivation of VHL (using siRNA) is indeed sufficient to result in such alterations in PPAR γ action in our proximal tubule cells.

Other Diseases I (731 – 754)

731/B696

Activity of Anthrax Lethal Toxin: Impact of the N-Terminal Amino Acid.

P. K. Gupta, M. Moayeri, S. H. Leppla; LBD, NIAID, Bethesda, MD

The virulence of *Bacillus anthracis*, causative agent of anthrax, depends in part on the production of anthrax toxin, composed of protective antigen (PA) and two catalytic moieties, lethal factor (LF) and edema factor (EF). PA is the central receptor-binding component which delivers LF and EF into the cytosol of mammalian cells. LF combined with PA is known as lethal toxin and is responsible for lethality in various cell types and animal models. LF is a zinc-dependent metalloprotease that cleaves several MAPK kinases. LF has been expressed from a variety of different host organisms, including *E. coli*, *B. subtilis* and *B. anthracis*. LF expressed in the past from non-toxigenic strains of *B. anthracis* in our laboratory had the two residues His-Met added at its N-terminus due to the cloning manipulations. In the present studies we observed that the presence of these two additional residues in recombinant LF decreased its potency for cultured cells and two animal models (mouse and rat) as compared to native LF. We hypothesized that the N-terminal residue of LF could determine the stability of the toxin in the cytosol of cells and thereby its potency. This hypothesis was based on the N-end rule of protein degradation which states that for certain proteins, the identity of the N-terminal amino acid residue determines the rate of cytosolic ubiquitinylation and thereby the rate of degradation by proteasomes. We produced mutant LF proteins having Gly, Arg, Phe, Met or His as N-terminus. LF with the stabilizing residue Met was the most toxic in both cell culture and two animal models. Addition of the potent destabilizing residue Phe to LF resulted in significantly decreased LF potency for both macrophages and rats, while other residues showed intermediate effects both *in vitro* and *in vivo*. The results were consistent with the hypothesis that the N-end rule applies to LF stability and potency. These data have implications for production and quality control analyses of LF since it is widely used in assays to characterize candidate vaccines and therapeutics for anthrax.

732/B697

Posphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) as a Key Mediator of Genome Replication for Hepatitis C Virus (HCV).

N. Cho, C. Lee, P. Pang, J. S. Glenn; Stanford University, Stanford, CA

PURPOSE: Hepatitis C virus (HCV) is a primary cause of liver disease and represents a significant medical problem. Treatments for this disease are inadequate and novel anti-HCV strategies are necessary. In this study, we sought to identify a novel role for posphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) as a key mediator of genome replication for hepatitis C virus (HCV) METHODS: We adapted the technique of quartz crystal microbalance with dissipation (QCM-D), wherein Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) containing polymerized liposome can be intacted on quartz crystal nanosensors and binding of peptide derived from NS5A are able to be studied in real time. In addition, PtdIns(4,5)P2 binding induced a significant conformational change in the AH detected by circular dichroism RESULTS: Polymerized liposome contained PtdIns(4,5)P2 are coated on an oscillating quartz crystal nanosensor and found significant binding of the NS5A AH to PtdIns(4,5)P2-containing liposome was observed. The QCM-D technique allows for ready determination of binding kinetics, and a Kd for the NS5A AH association with PtdIns(4,5)P2-containing liposome was found to be 4.365 uM. To determine the specificity of the observed binding, a polymerized liposome containing PtdIns(3,4)P2, PtdIns(3,5)P2, or no PtdIns were used in parallel assays and identify that specificity observed for NS5A AH binding to PtdIns(4,5)P2. In addition, CD measurements of the NS5A AH peptide were performed in the presence or absence of PtdIns(4,5)P2 in which dramatic alteration of the helical structure of the AH was observed upon interaction. Therefore, PtdIns(4,5)P2 binding appears to mediate a conformational change in NS5A, and we hypothesized that this represents an interaction that is essential for replication CONCLUSIONS: Our results suggest a novel role for Phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2) as a key mediator of genome replication for hepatitis C virus (HCV). These results demonstrate the first example of phosphoinositides-mediating viral genome replication, suggest the potential for novel anti-HCV strategies.

733/B698

The N-terminal Regions of the HPS1 and HPS4 Proteins are Required to Form the Biogenesis of Lysosome-related Organelle Complex 3 (BLOC-3).

C. Carmona[1], J. Bonifacino[2], C. Cadilla[3], W. A. Gahl[1]; [1]National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, [2]National Institutes of Child Health and Human Development, National Institutes of Health, Bethesda, MD, [3]Biochemistry, University of Puerto Rico, School of Medicine, San Juan

Hermansky-Pudlak Syndrome (HPS) [MIM#203300] is a rare autosomal recessive disorder characterized by oculocutaneous albinism, a bleeding tendency, and sporadic pulmonary fibrosis, granulomatous colitis of infections. HPS is a heterogeneous disorder; eight HPS-causing genes have been identified in humans. HPS type 1 is the most severe subtype with a prevalence of ~1/1800 in Northwest Puerto Rico. Mutations in HPS genes affect the biogenesis of lysosome-related organelles such as melanosomes and platelet dense granules. The products of the HPS type 1 and 4 genes (i.e., HPS1 and HPS4) assemble to form a complex known as Biogenesis of Lysosome-related Organelle Complex 3 (BLOC-3). Here, we report the identification of two novel splice forms of HPS1, called variants 5 (HPS1ss1) and 6 (HPS1ss3). We found that HPS1ss3 is able to assemble with HPS4 whereas HPS1ss1 is not. These findings, in combination with the results of truncation analyses, allowed us to identify a region spanning amino acids 1-300 that is required for HPS1-HPS4 assembly. In addition, HPS4 displays a dual binding pattern comprised by its N-terminus (1-230) and a discrete region between residues 340-399. The N-terminus of HPS4 interacts with the first 300 residues of HPS1 and the discrete region of 340 to 399 HPS4 amino acids showed an interaction with an area between residues 300-575 of the HPS1 protein. Our findings correlate with the CHiPS domain identified in HPS-4/Ccz1 by previous bioinformatic analysis. The identification of the regions involved in the HPS1 and HPS4 interaction represent an important first step in the mapping of the domains that sense regulatory signals responsible for the biogenesis of lysosome-related organelles. These observations extend our

understanding of BLOC-3 assembly. This work was partially supported by the IRTA-NIH Program and grants R25GM81838 and RCMI G12RR03051.

734/B699

Cellular Development in a Mouse Model for Lung Fibrosis.

L. Wang, T. Lyerla; Biology Department, Clark University, Worcester, MA

Lung fibrosis is a complicated cellular disease involving TGF beta-1 signaling, inflammatory alveolar macrophages (AMs) and apoptosis. In order to determine possible interactions of these factors, we studied their time course in the mouse model C57BL/6-*Hps1*^{ep} *Ap3b1*^{pe}-J for Hermansky-Pudlak Syndrome (HPS). This strain possesses abnormal alveolar type II (ATII) cells and develops lung fibrosis naturally at about 17 months. The metabolic activities of AMs in bronchial alveolar lavage (BAL) samples were tested using Alamar blue. TGF beta-1, a key cytokine for pulmonary fibrosis, was localized by IHC and quantified by ELISA. Apoptosis was tested by IHC using cytochome C and Bax as markers. ATII cells were labeled with prosurfactant C antibody. Wild type strain, C57BL/6J-+/+, and the beige mutant mouse strain, C57BL/6-*Lyst*$^{bg-J}$-J, with abnormal ATII cells, were used as controls. AM individual cell activities and TGF beta-1 levels remained higher than controls in BAL from HPS mice throughout their life times, and started to exhibit increased levels after about 9 months of age. Greater numbers of ATII cells in HPS mice were positive for apoptosis markers at all ages compared with controls, and fibrotic patches started to invade epithelial areas in HPS mice after one year of age. IHC staining with TGF beta-1 labeled the AMs and some ATII cells, similar to the staining of apoptotic markers. These results showed that lungs from postnatal HPS mice were abundant with highly activated AMs and with ATII cells susceptible to apoptosis throughout their life span. At about one year of age, these abnormalities accelerated in levels when initial stages of lung fibrosis became apparent histologically, presumably due to the break down of homeostatic mechanisms protecting younger HPS mice. Partial support for these studies was provided by NIH grant 1R15HL091503-01 and it is gratefully acknowledged.

735/B700

A Polycystin-1 Interacting Protein Functions in Regulating Ciliagenesis in Kidney Epithelial Cells.

G. Yao[1], K. Roberts[1], X. Li[1], A. Takakura[1], M. Plomann[2], J. Zhou[1]; [1]Renal Division, Brigham and Women's Hospital and Harvard Medical School, Boston, MA, [2]Center for Biochemistry, University of Cologne, Cologne, Germany

Polycystin-1 plays an essential role in renal tubular morphogenesis, and disruption of its function causes cystogenesis in human autosomal dominant polycystic kidney disease (ADPKD). In order to identify polycystin-1 interacting proteins, we performed yeast two-hybrid screens with the polycystin-1 C-terminus as bait, and we isolated an isoform of pacsin protein family, PAC. PAC has a function in regulating the dynamin-mediated endocytotic process and cell skeleton organization. Coimmunoprecipitation experiments verified the physical interaction between polycystin-1 and PAC. By yeast co-transformation assay, we located the polycystin-1 binding domain is from a.a. 171- a.a. 278 of PAC. Indirect double immunofluorescence experiments showed that PAC co-localizes with PC1 on the primary cilium of not only IMCD3 cells, but also in normal human kidneys. Knockdown of PAC by shRNA affects the length of primary cilium and causes accumulated polycystin-1 in IMCD3 cells, especially on the tip of the primary cilium. Real-Time PCR indicates that knockdown of PAC by shRNA did not alter the steady state levels of Pkd1 mRNA. However, by western analysis, we found an increase in polycystin-1 expression levels. Recent studies suggested that polycystin-1 could be secreted into the urine exosomes. By immunoelectron microscope, we found both polycystin-1 and PAC are present in the isolated exosomes, which suggests that PAC may regulate polycystin-1 exosomal secretion. PAC may contribute to cystogenesis in ADPKD through interaction with polycystin-1 and regulating polycystin-1 stability and the length of the primary cilium of epithelial cells.

736/B701

Developement of Delivery System for Mycobacterial Antigen ESAT-6.

S. Chandra, M. Kaur, R. Bhatnagar; School of Biotechnology, Jawaharlal Nehru University, New Delhi, India

Development of vaccines with the ability to generate specific Cytotoxic T lymphocyte (CTLs) is hindered due to paucity of delivery systems of CTL epitopes into the cytosol of host cells. Delivery into cytosol and presentation through MHC I molecule is a necessary step for elicitation of CTLs against the host cells expressing foreign peptide. ESAT-6 is an important T-cell antigen of *Mycobacterium*, due to its relatively low inherent immunogenicity, efforts are directed towards enhancing its ability to evoke a robust T cell response, crucial in the context of tuberculosis infection. In this study, we report the ability of N-terminal fragment of both lethal factor and edema factor to deliver genetically fused ESAT-6 (early secretory antigen target), a potent T cell antigen of *Mycobacterium tuberculosis*, into cytosol of mammalian cells to elicit CTL response. EFn.ESAT-6 and LFn.ESAT-6 recombinant protein were obtained by genetic fusion of EFn - ESAT-6 and LFn - ESAT-6 DNA respectively. Our data shows that in the presence of PA, EFn.ESAT-6 and LFn.ESAT-6 fusion proteins are internalized into the cytosol of antigen presenting cells, and the splenocytes produce both Th1 and Th2 cytokines in vitro. Since CTL response is essential for protection against intracellular pathogens and, it is well known that only single T cell epitope or single antigenic protein is not sufficient to elicit protective CTL response due to variation or polymorphism in MHC-I alleles among the individuals, we suggest that as a fusion protein LFn and EFn can be used to deliver multiepitopes of T cells or multiproteins which can generate effective CTLs against intracellular pathogens like *M. tuberculosis*. Such delivery systems can also be utilized for other pathogens for enhancing their delivery and clearance.

737/B702

Anti-oxidative and Anti-atrophic Effects of a Highly Soluble, Acid Stable UDCA Formula in AGS Cells and Helicobacter pylori Induced-Gastritis.

T. D. Tran, D. Rhee; Pharmacy, Sungkykwan University, Suwon, South Korea

H. pylori is a gram-negative bacillus that colonizes the gastric mucosa and produces superoxides and other products such as Vac and Cag, which cause DNA damage and induce cytokines and growth factors that contribute to inflammation and chronic gastritis as well as promote gastric carcinogenesis, the second most common cancer in the world. Ursodeoxycholic acid (UDCA) has been used to treat a variety of liver diseases such as primary biliary cirrhosis, primary sclerosing cholangitis, and cystic fibrosis-related cholestasis. However, UDCA products

currently available have low solubility and stability in acidic pH, and do not inhibit the colonization of *H. pylori*. Here, effects of a highly soluble and pH stable UDCA formula on oxidative damages in AGS cells as well as mice were investigated. The results showed that UDCA has a different effect on TCA cycle enzymes in a normal and gastritis model. It increased malate dehydrogenase (MDH) activity in normal AGS cells *in vitro* but decreased MDH activity in *H. pylori*-induced gastritis mice. Moreover, UDCA also increased the mitochondria membrane potential both *in vivo* and *in vitro*, thereby preventing the depletion of ATP from the mitochondria in a *H. pylori*-infected mouse stomach. It also could scavenge respiratory oxygen species (ROS) and reduce apoptosis in H2O2- and *H. pylori*-induced apoptosis. In conclusion, in this study, we reported for the first time that the new UDCA formula could scavenges ROS and increases mitochondrial membrane potential, which prevents H2O2-induced apoptosis through the mitochondria-mediated pathway in AGS cells.

738/B703

Cell Culture Studies to Elucidate the Role of γ-Actin Mutations Causing Non-Syndromic Hearing Loss.

S. Korrapati, K. Friderici; Michigan State University, East Lansing, MI

Mutations in the γ isoform of actin cause non-syndromic, post-lingual, autosomal-dominant, progressive sensorineural hearing loss. The two cytoplasmic actins (β,γ) are identical to each other except at 4 amino acid positions in the amino terminus. Localization studies of the actins in guinea pig and chicken cochlear hair cells reveal differential spatial arrangements of the two isoforms. Our hypothesis is that γ-actin is involved in specific functions in the inner ear. Stereocilia are actin-filled structures found on the apical surface of hair cells. These are derivatives of actin-filled microvilli found on the surface of many cell types. The filamentous actin in the stereocilia is bundled by fimbrin and espin. The pig proximal kidney epithelial cell line, LLC-PK1-CL4 (CL4), lacks endogenous espin, but cells form long spiky microvilli when transfected with espin. Using CL4 cells as a model system, our goal is to study the function of actin isoforms and investigate the role of γ-actin and its mutants in the microvilli. The results obtained by confocal imaging of CL4 cells indicate that the cytoplasmic actins are differentially distributed in the cell. β-actin is located primarily at the periphery while γ-actin is abundant in the perinuclear space and cytoplasm of the cell. Exogenous expression of β and γ-actins reveals distribution of actins to all the relevant actin rich structures in the cell. In response to exogenous espin expression, espin and filamentous actins (β,γ) co-localize in microvilli and co-transfection of espin and the actin mutants resulted in each of the mutants co-localizing with actin in microvilli. To characterize the role of actins and mutants in the microvilli, stable GFP-tagged cell lines of espin, wild type actin and mutants were constructed. The data obtained thus far suggest that when mutant actin is present in espin expressing cells, the resulting microvilli are shorter than with wild type. This could support the suspicion that mutations in γ-actin interfere with basic processes required for stereocilia maintenance. Studies are currently underway to understand the cause of the short microvilli.

739/B704

Modulation of AlphaV Beta 3 Integrin Expression in a Rat Model of Ulcerative Colitis.

J. Saliba[1], S. Ghanem[1], I. Abdallah Hajj Hussein[1], R. Saadeh[1], R. Jurjus[2], M. Stepp[2], J. Freund[3], A. Jurjus[1]; [1]Human Morphology, American University of Beirut, Beirut, Lebanon, [2]Anatomy and Regenerative Biology, George Washington University, Washington DC, WA, [3]Universite Louis Pasteur, Strasbourg, France

Background: Ulcerative colitis (UC) is associated with inflammation of the colonic layers, mainly the mucosa and the submucosa. Various types of integrins were reported to be involved in colitis and colonic tumors, in particular the alphaVbeta3 integrins. The aim is to assess the modulation of expression of this integrin complex in UC, in our experimental rat model. Methods: The present model of experimental colitis was induced in 20 adult Sprague-Dawley rats by the injection of a chemical (iodoacetamide) followed by bacterial inoculation. The model included also a control group of 20 animals treated with methylcellulose which is the vehicle for the dilution of the iodoacetamide. Colitis was assessed based on clinical aspects of the disease (diarrhea, weight loss, etc) as well as molecular changes (increased expression of inflammatory cytokines) and histological examination of the descending colon (disruption of mucosa, crypt abnormalities, inflammatory cells infiltrates, etc). Expression of αVβ3 integrins was assessed by indirect immunofluorescence using a monoclonal antibody against the αVβ3 complex, on 5µm-thick paraffin sections. Results: Examination of the FITC-labeled duplicate sections showed fluorescence lining blood vessels in the colonic lamina propria and spots scattered along the base of the mucosa. Control sections (obtained from biopsies of the control group rats) showed significantly less fluorescence. The group treated with IA+B combined showed highest expression of αVβ3 integrins on day 28 of the experiment. Conclusion: The results showed that chronic ulcerative colitis is associated with higher levels of αVβ3 integrin putting the colon at risk of developing tumor masses supplied by newly forming blood vessels.

740/B705

Evaluation of the Common Variants of the ABCA4 Gene in Families with Stargardt Disease and Autosomal Recessive Retinitis Pigmentosa.

B. Shastry; Biological Sciences, Oakland University, Rochester, MI

Stargardt disease (STGD) is one of the most common autosomal recessive retinal dystrophies that has an estimated incidence of one in 10,000. It affects the central retina (macula). Retinitis pigmnentosa (RP) comprises a large and exceptionally heterogeneous group of hereditary disorders of the retina. It is caused by the loss of photoreceptors and affects both eyes. The condition is characterized by retinal pigment deposits and has an estimated incidence of 1:4000. Recently, the photoreceptor cell-specific ATP binding cassette transporter (ABCA4) gene was found to be mutated in patients with STGD as well as autosomal recessive RP. In order to understand further the contribution of this gene to the susceptibility to the STGD and RP, we have analyzed three unrelated STGD and one autosomal receive RP families specifically for the more common variants (A1038V, G1961E, 2588 G to C, R943Q or 2828 G to A) in the ABCA4 gene. Our analyses employing standard techniques such as polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP), and direct DNA sequencing of amplified products have been able to identify one common variant (R943Q) in all three STGD families but not in RP family. All three affected STGD individuals however, are heterozygous for this variation and this alteration did not segregate with the disease and is also present in normal controls. Similar analysis of other common variants did not reveal any pathogenic mutations in both STGD and RP families except in the case of STGD rare intronic polymorphisms in exon 21 and exon 47 have been identified in both affected

and normal controls. It is likely that the variant identified in this study represents a rare polymorphism (non-pathogenic). Future extensive studies on this as well as other candidate genes may uncover the susceptibility gene for these recessive forms of the disorders in these families.

741/B706

The Phosphorylation State of the Fragile X Mental Retardation Protein Affects Its Association with Dicer.

A. Cheever, S. Ceman; Cell and Developmental Biology, University of Illinois, Urbana-Champaign, IL

The fragile X mental retardation protein, FMRP, is an RNA binding protein that regulates translation of associated mRNAs. Phosphorylation of FMRP has been implicated in translational suppression on polysomes and of one mRNA ligand [1,2]. Our goal is to elucidate how phosphorylation of FMRP regulates translation of its bound mRNAs. We hypothesize that translational regulation by phosphorylated FMRP (P-FMRP) might be accomplished through association with the microRNA (miRNA) pathway. FMRP is found in a complex with RISC, Ago2, Dicer, and other proteins associated with the miRNA pathway. Dicer processes precursor (80 nucleotide) miRNAs into functional 21-23 nucleotide miRNAs that are inserted into the RISC complex. Association with miRNAs may lead to either suppression or activation of mRNA translation depending on associated proteins in the miRNP complex [3]. We have developed and characterized both a phospho-specific antibody to P-FMRP and an antibody that detects FMRP regardless of its phosphorylation state. Using these antibodies we show that phosphorylated FMRP associates with significantly increased amounts of a 70-80 nucleotide species, which we suspect is precursor miRNA, compared to total FMRP. Further, we show that P-FMRP is unable to associate with Dicer in co-immunoprecipitation experiments and a peptide capture assay. Thus, translational suppression by P-FMRP does not appear to be through enhanced miRNA binding. Our data suggest the phosphorylation state of FMRP is an important factor in its association with Dicer, a key component of the miRNA pathway, suggesting a novel function for P-FMRP. 1. Ceman S, et al. Hum Mol Genet. 2003 Dec 15; 12 (24): 3295-305. 2. Narayanan U, et al. J Neurosci. 2007 Dec 26; 27 (52): 14349-57. 3. Vasudevan S, et al. Science. 2007 Dec 21; 318 (5858): 1877-8.

742/B707

Pro- and Anti-apoptotic Pathways in Experimental Nephropathy.

Q. Guo, J. Xie; Department of Physiology, the University of Oklahoma Health Sciences Center, Oklahoma City, OK

Par-4 (prostate apoptosis response-4) is a leucine zipper protein of about 38 kDa that plays an important role in regulating apoptosis. The par-4 gene has been mapped to human chromosome 12q21. We reported Par-4 regulates survival and death of renal tubular epithelial cells following ischemia/reperfusion induced injury (IRI). Functional studies indicated that Par-4 expression was necessary to induce apoptosis of renal tubular epithelial cells, since blockade of Par-4 activity (by co-expression of a dominant negative regulator of par-4 or RNA interference) prevented apoptosis. AATF (apoptosis antagonizing transcription factor) was another leucine zipper protein which was initially identified as an interaction partner of DAP-like kinase (Dlk), a member of the DAP (Death Associated Protein) kinase family of pro-apoptotic serine/threonine kinases. We have found that AATF binds with Par-4 via the leucine zipper domain, leading to a complete blockade of the pro-apoptotic signaling mediated by Par-4. We now provide evidence that AATF is a phosphorylation substrate of Akt1/PKBα, a serine/threonine protein kinase and an important downstream target of phosphatidylinositol 3-kinase (PI 3-kinase). Furthermore, phosphorylation of AATF by Akt1 regulates the renoprotective activity of AATF in renal tubule cells following IRI.

743/B708

Proteomic Analysis of Airway Lining Fluids in Asthma.

C. Park, J. Kwon, S. Park, K. Kim, Y. Lee, J. Park, T. Rhim; Soonchunhyang Univ. Bucheon Hospital, Bucheon, South Korea

Bronchoalveolar lavage fluid (BALF) is presently the most common way of sampling the components of the epithelial lining fluid and of faithfully reflecting the protein composition of the airways. Thus, proteomic analysis of BALF may be important for understanding the pathobiology of asthma at the protein levels. In this study, tried to identification of differentially expressed proteins in BALF from asthmatics and normal controls. We compared the BALF protein profiles of asthmatics (n=8) and healthy normal subjects (n=8) on 2-DE analysis. The differentially expressed protein spots were identified by using MALDI-TOF MS and LC-MS/MS. Among them, apolipoprotein A-1 (APOA-1) was validated using conventional ELISA. In addition, effect of APOA-I on asthma development was evaluated in OVA sensitized/challenged asthma mice model. 16 proteins were found to be differentially expressed in BALF in the two groups. MALDI-TOF MS and LC-MS/MS demonstrated that Vitamin D binding protein, Haptoglobin β, Hemopexin, Protein disulfide-isomerase A3 and Microtubule-actin crosslinking factor 1 were increased in BALF of asthmatics, while α1-antitrypsin and apolipoprotein-AI were down-regulated. The concentrations of APOA-I measured by ELISA were lower in BALF of asthmatics (n=54, 10.33±1.07ug/ml) than those of normal subjects (n=18, 22.17±5.01ug/ml) (p=0.003). Intratracheal treatment of APOA-I in OVA sensitized/challenged asthma model markedly decreased the airway hyperresponsiveness, and inflammatory cell counts and IL-4, IL-5, IL-13 and eotaxin levels in BALF. The proteomic analysis of BALF indicates that alternation of inflammatory/anti-inflammatory proteins in the airway may contribute to asthma development and APOA-I may be one candidate useful for the treatment of asthma. This study was supported by the grants of the Korea health 21 R&D project (Ministry of Health, welfare and family Affairs, Republic of Korea, A030003)

744/B709

Heparin Augmentation of TPA-induced Osteoclastic Differentiation of THP-1 Monocytic Cells: Involvement of MEK/ERK and JAK/STAT Signaling.

K. Matuoka, K. Sasaki; Pharm. Sci., Chiba Institute of Science, Chiba-ken, Japan

We reported previously that HL60 promyeloblastic cells differentiated into osteoclastic cell types by a combination of 12-O-tetradecanoylphorbol-13-acetate (TPA) and 1α,25-dihydroxyvitamin D3 (VD3). The changes were augmented in the presence of heparin (HP), a glycosaminoglycan species known to cause osteoporosis upon long-term clinical administration. Here, in order to investigate molecular systems underlying the osteoclast formation and the HP action, we employed other monocytic cell stains THP-1 and U937. When exposed to TPA or TPA+VD3 for 3 days or longer, the cells became adherent with some subpopulations being flattened, enlarged and/or

multinucleated or elongated. Expression of tartrate-resistant acid phosphatase activity, an osteoclast marker, was also induced. Unlike HL60 cells, TPA alone could induce the changes in THP-1 and U937 cells, although TPA+VD3 were more potent. Next, cells were pretreated with HP for 24 h before exposure to TPA+VD3. The results showed that HP increased those morphological changes as well as the number of those adherent cell populations. That might suggest HP enhancement of osteoclast formation/activity in the bone remodeling. In an attempt to analyze the signaling events involved in the osteoclastic differentiation, THP-1cells were given kinase inhibitors LY294002 (against PI3K), PD98059 (against MEK/ERK), SB203580 (against p38 MAPK), SP600125 (against JNK/SAPK) and AG490 (against JAK/STAT) 6 h prior to TPA+VD3. LY294002 caused a decline in the number and size of adherent cells, conceivably due to apoptotic loss of the differentiation-induced cell population. PD98059 decreased the enlarged cells as well as long-shaped cells, which might be an indication that the cellular differentiation process was suppressed. With regard of the HP actions, most kinase inhibitors failed to abolish the phentypic increments due to HP; however, in AG490-treated cultures HP could not enhance the cellular changes. Taken together, possibilities have been suggested that TPA+VD3-promoted monocytic cell differentiation into osteoclastic phenotypes substantially rely on MEK/ERK system and that HP augments these changes through additional utilization of JAK/STAT signaling.

745/B710

TGF-β1 Induces Epithelial to Mesenchymal Transition of Hepatocytes, Which Results In Hepatocyte Dysfunction and Collagen Production.

J. Wang, J. Han, J. Kim, M. Kim, J. Lee, W. Kim; Ajou University School of Medicine, Suwon, South Korea

Background/Aims: Liver cirrhosis is characterized by hepatic dysfunction with fibrosis. We addressed whether TGF-β1 induces epithelial to mesenchymal transition (EMT) of hepatocytes, and also whether the EMT contributes to both hepatocyte dysfunction and liver fibrogenesis. Methods: We assessed EMT by immunocytochemistry and Western blotting. Hepatocyte function was measured by albumin expression and ammonia-metabolizing activity. Collagen production was measured by RT-PCR. Immunohistochemistry was performed to assess EMT in tissues. Results: TGF-β1 induced EMT of primary rat hepatocytes, evidenced by decrease of epithelial markers such as cytokeratin18 and ZO-1, cytosolic translocation of E-cadherin, as well as increase of mesenchymal markers such as N-cadherin, vimentin and α-SMA. EMT of hepatocytes was also observed in DMN-induced rat liver fibrosis model. Interestingly, hepatocytes which underwent EMT showed type I collagen expression, suggesting the contribution of EMT to liver fibrogenesis. In addition, TGF-β1-treated hepatocytes and hepatocytes isolated from fibrotic liver revealed a significant decrease of hepatocyte function, assessed by ammonia-metabolizing activity and albumin production. Furthermore, human cirrhotic liver tissues revealed changes similar to those observed in rat liver fibrosis model, suggesting that EMT of hepatocytes is present both in rat and human. Conclusions: EMT of hepatocytes via TGF-β1 contributes to both hepatocyte dysfunction and liver fibrosis.

746/B711

Azithromycin Induces Autophagic Cell Death in H9c2 Cells.

E. Seo[1,2], M. Kim[1,3], E. Kim[4], H. Kim[5], H. Cho[5], E. Chung[5], S. Lee[1,2], E. Baik[1,2], C. Moon[1,2], Y. Jung[1,3]; [1]Department of Physiology, Ajou University, Suwon, South Korea, [2]Brain Korea 21 for Medical Sciences, Ajou University, Suwon, South Korea, [3]Brain Korea 21 for Molecular Science and Technology, Ajou University, Suwon, South Korea, [4]Korea Institute of Toxicology, KRICT, Taejon, South Korea, [5]Korea Food and Drug Administration, Seoul, South Korea

Azithromycin, an azalide subclass macrolide antibiotics, has been used worldwide in the treatment of respiratory infections including pneumonia. Recently, however, its utilization has been alerted due to its capability of QT prolongation and life-threatening proarrhythmic potential. Although azithromycin was demonstrated to induce apoptotic cell death in lymphocytes, there is no information about its ability of inducing cardiomyocyte death. In the present study, we investigated whether azithromycin could induce apoptosis and/or autophagic cell death in rat heart-derived H9c2 cells as a mechanism of reduced cell viability. Cell viability was determined by MTT assay and morphologic change was observed by transmission electron microscopy. Azithromycin decreased the cell viability in a dose-dependent manner. In the azithromycin-treated cells, numerous autophagosomes were observed, while any cytoplasmic vacuoles in control cells were not observed. Bafilomycin A1, a specific autophagy inhibitor, suppressed the development of cytoplasmic vacuolization caused by azithromycin and it also recovered azithromycin induced reduction in cell viability. Azithromycin-induced autophagy was confirmed by the LC3 conversion. Azithromycin elicited significant increase in the levels of LC3 II, compared with the control. However, the TUNEL-positive cells were hardly found in azithromycin-treated cells. Caspase inhibitors did not prevent the reduction of cell viability caused by azithromycin. And caspase-3 activity remained unaltered in azithromycin-treated cells. In conclusion, these results suggest that azithromycin induces autophagic cell death rather than apoptotic death. This work was supported by grant from the Korea Food and Drug Administration.

747/B712

Caspases Pathway a Therapeutic Target in Pemphigus.

D. Pacheco-Tovar, A. Lopez-Luna, J. Bollain-y-Goytia, R. Herrera-Esparza, E. Avalos-Diaz; Immunology. Centro de Biologia Experimental, Universidad Autonoma de Zacatecas, Zacatecas, Mexico

Introduction. Pemphigus is an autoimmune disease characterized by the development of intraepidermal blisters. These lesions are caused by anti-desmoglein autoantibodies which bind the desmosome structure and induce cell detachment (acantholysis), which is followed by apoptosis. Pemphigus autoantibodies are pathogenic, because the injection of human pemphigus IgG into Balb/c neonatal mice induces blisters. Theoretically the blockage of caspases pathway could prevent the blistering caused by pemphigus autoantibodies. Objective. Present investigation attempts to block the pathogenic effect of the pemphigus IgG in Balb/c mice by caspase 3inhibition. Methods. A serum from pemphigus vulgaris patient with anti-desmoglein 1 and 3 antibodies (ELISA), was used to purify IgG by affinity chromatography (HiTrap protein G HP). The IgG fractions were concentrated to 100 mg/ml and filter sterilized. Dose was adjusted to 10 mg of IgG per gram of body weight, and it was given intraperitoneally to Balb/c neonatal mice (n=5/group). Another group of animals received the MDEVD-CMK caspase 3 blocker before the injection of pemphigus IgG. The presence of blisters in experimental animals was evaluated in the following 24 hours. Skin biopsies were evaluated by direct immunofluorescence, and the presence of phosphatidylserine on the outer leaflet of apoptotic

membranes was detected by Annexin-V-FLUOS staining, and .the apoptotic DNA breaks were detected by TUNEL. Results. The injection of pepmhigus IgG into Balb/c neonatal mice induced intraepidermal blisters. Blistering was located in basal epidermis, therefore pemphigus IgG was found deposited along desmosomes of lesional and perilesional areas. Interestingly the pemphigus IgG deposition induced acantholysis and apoptosis. The caspase 3 blocker DEVD-CMK avoids apoptosis and prevents the blisters formation. Conclusions. Present work demonstrate that the blocking of caspase 3, prevent the blister formation in experimental pemphigus. This proposal can be applied in clinical practice to control the flare-ups of pemphigus.

748/B713

Readthrough-inducing Ointment: the New Approach for the Treatment of Nonsense Mutation-Mediated Disorders.

M. Shiozuka[1], T. Kawamoto[2], H. Sasaki[3], K. Shimada[1], A. Wagatsuma[1], Y. Nonomura[4], R. Matsuda[1]; [1]Dept. of Life Sci., The Univ. of Tokyo, Tokyo, Japan, [2]RI Research, Tsurumi Univ., Yokohama, Japan, [3]DNA Medicine, The Jikei Univ., Tokyo, Japan, [4]Microbial Chemistry Research Foundation, Tokyo, Japan

The ability of aminoglycoside antibiotics, such as gentamicin, to promote readthrough of premature termination codons has attracted interest in these drugs as potential therapeutic agents in nonsense mutations. In order to measure readthrough efficiency with quantitative accuracy, we have established transgenic mouse strain containing dual-reporter gene composed of beta-galactosidase and luciferase connected with premature termination codon region. The objective of this study was to evaluate the transdermal drug delivery (TDD) potential for treatment of genetic diseases caused by nonsense mutations. TDD is an appealing alternative that offers good patient compliance and the possibility of controlled release over time while avoiding possible degradation due to the gastrointestinal tract or first-pass liver effects. Despite these advantages, TDD is severely limited by the low permeability of skin caused mainly by stratum corneum. Here, we show that skin permeability can be increased through the use of chemical enhancer. It was found that the readthrough activity by transdermal administration of gentamicin resulted similar to those by subcutaneous injection. To evaluate in vivo permeation of gentamicin in serum or muscle tissue from hairless mice, as measured with liquid chromatography-tandem mass spectrometry. Application of chemical enhancer to the skin increased its permeability and enabled the delivery of gentamicin through the skin. Further, the expansion of the intercellular gaps in the prickle cell layer was observed by electron microscopic analysis of the skin. These readthrough therapy on TDD may provide significant value in treating or preventing genetic diseases associated with nonsense mutations. This work was supported by a Research Grant for Nervous & Mental Disorders and by a Grant for Research in Brain Science from the Ministry of Health, Labor and Welfare, Japan.

749/B714

Corneal Light Absorption Increases along the Irradiation of the Cornea with UVB Rays.

C. Cejka, J. Platenik, T. Ardan, J. Cejkova; Department of Eye Histochemistry and Pharmacology, Institue of Experimental Medicine, Prague 4, Czech Republic

The eye and particularly the cornea are directly exposed to UV rays from sunshite. The cornea absorbs and detoxifies the majority of UVB rays (approximately 80%) and thus it acts as a UVB filter. These processes are ensured by corneal tissue components and fluids, of which low molecular weight and high molecular weight antioxidants are highly involved. Purpose of this study was to examine light absorption properties of the cornea repeatedly irradiated with UVB rays. In the first group of rabbits the corneas were irradiated with UVB rays (312 nm, once a day during 4 days, a dose per day 1.01 J/cm2). The animals were sacrificed one day after the last irradiation. The rabbits of the second group were similarly irradiated, however after the last irradiation they were left without any treatment for ten days and afterwards they were sacrificed and corneas examined (spectrophotometrically, histologically, biochemically and immunohistochemically for malondialdehyde and nitrotyrosine). Absorbances of corneal centers (normal and irradiated corneas) were measured over a range of 190-650 nm using a scanning spectrophotometer (HELIOSb 84021). Results show that corneal light absorption increases along the repeated irradiation with UVB rays (from UV to visible light region). This was due to the increased corneal thickness and slight changes of corneal transparency. The corneal changes returned to normal values during ten days following the last irradiation. In conclusion, the amount of UVB absorbed in the cornea highly increased due to repeated irradiation. It is suggested that the increased corneal light absorption contributes to the protection of the inner eye against the damaging effect of UVB rays and reactive oxygen products induced by them (important for animals living outdoors).

750/B715

Mechanisms Involved in Inflammatory Events Induced by Venom of Brown Spider Loxosceles Intermedia and its Recombinant Dermonecrotic Toxin LiRecDT1.

K. S. Paludo, Y. B. Sade, C. R. Franco, S. S. Veiga; Cell Biology, Federal University of Paraná, Curitiba, Brazil

Acidents involving Brown spider venom produce necrotic skin degeneration, gravitational spreading and massive inflammatory response at injured region. This venom has a complex mixture of different toxins, and the dermonecrotic toxin is the major contributor to toxic effects. The ability of Loxosceles intermedia whole venom and a recombinant isoform of dermonecrotic toxin (LiRecDT1) to induce edema and increase in vascular permeability; the investigation of mechanisms involved in these responses and the report of direct action on endothelial cell surface and extracellular matrix were the objetives of this work. Whole venom and LiRecDT1 were injected into hind paws and the change in thickness over time (for edema) and the leakage of plasma protein-bound dye into the extravascular compartment of the skin (for vascular permeability) was measured. These venom toxins cause a marked dose and time-dependent edema and increase in vascular permeability in mice. Furthrermore, the enzimatic acitivity of venom toxins may be primal for these efects. A mutated recombinant isoform of dermonecrotic toxin (LiRecDT1 H12A) (that have just residual enzimatic activity) was not able to induce these inflammatory events. In additon, the ability of venom toxins to evoke these inflammatory events was partially reduced in compound 48/80 pretreated animals, suggesting that mast cells may be involved in these responses. Whole venom was able to causes mast cell degranulation in vitro reinforcing this suggestion. Morever, HLPC analysis of the whole venom showed the presence of histamine sufficient to induce inflammatory responses. Besides, we have performed immunofluorescence and flow cytometric analysis. In these experiments venom toxins are able to bind endothelial cell surface and extracellular matrix, inducing disorganisation of cell focal adhesion points and actin microfilaments associated to a change in fibronectin matrix profile. In conclusion, the inflammatory events induced by L. intermedia toxins may result by activation of

mast cells which in turn release vasoactive amines, by the histamine content in venom and could be triggered by deleterious effects of toxins on endothelial cell surface and extracellular matrix components.

751/B716

The Essential Function of Nox4-derived ROS in PDGF-induced MUC8 Overexpression in Airway Epithelial Cells.

H. Kim[1], J. Kang[2], U. Moon[3, 4], J. Yoon[2, 3, 5]; [1]Otorhinolaryngology, Chung-Ang University, Seoul, South Korea, [2]Department of Otolaryngology, Yonsei University College of Medicine, Seoul, South Korea, [3]The Airway Mucus Institute, Yonsei University College of Medicine, Seoul, South Korea, [4]BK21 project for Medical Science, Yonsei University College of Medicine, Seoul, South Korea, [5]Research Center for Natural Human Defense System, Yonsei University College of Medicine, Seoul, South Korea

Introduction: Platelet-derived growth factor (PDGF), can generate reactive oxygen species (ROS) and ROS may participate in the pathogenesis of various inflammatory diseases. NADPH oxidase (Nox), have unique function of generating ROS. Until now, MUC5AC and MUC5B are recognized to be the major airway mucin genes. However, MUC8 may be one of important mucin gene expressed in the chronic inflammation of human airway epithelium. Objective: The purpose of this study was to identify which mucin genes are induced by stimulation with PDGF and the enzymatic mechanism by which these genes are up-regulated in normal human nasal epithelial (NHNE) cells and sinusitis patients. Methods: mRNA expression of mucin genes and Nox subtypes was studied by real-time PCR. Intracellular ROS assay using confocal microscope was performed. Western blot analysis was used to examine the protein expression of PDGF and Nox in sinusitis patients. For investigation of specific function of Nox subtypes, we used siRNA of each Nox subtypes and recombinant adenovirus encoding Nox4 construct. Results:The expression of PDGF was increased in the tissue of sinusitis patients, comparing with normal subjects. After treatment of PDGF, we observed that MUC8 gene expression and the generation of ROS were increased and also suppressed by antioxidant, N-acetyl-L-cysteine through real time-PCR and intracellular ROS assay in NHNE cells. We observed that Nox4 plays a critical role in PDGF-induced intracellular ROS generation. Knockdown of Nox4 expression with Nox4 siRNA, decreased PDGF-induced intracellular ROS and MUC8 gene overexpression. Overexpression of Nox4 with AdNox4 increased intracellular ROS and MUC8 gene expression in NHNE cells. In addition, the expression of Nox4 was also increased in the tissue of sinusitis patients. Conclusions: We thought that PDGF and Nox4 plays an essential role in MUC8 gene expression and may be correlated with the mechanism of chronic sinusitis through intracellular ROS generation. We suggest that reduction of excessive intracellular ROS or inhibition of Nox4, an enzymatic source of ROS generation in NHNE cells may be an important therapeutic approach against chronic sinusitis.

752/B717

The Extract of Crinum asiaticum var. japonicum Stimulates Hair Growth.

J. Kang, S. Kim, J. Hyun, M. Kim, H. Boo, E. Yoo, H. Kang; Department of Pharmacology, School of Medicine, Institute of Medical Sciences,Cheju National University, Jeju, South Korea

Objective: This study was conducted to evaluate the effect of *Crinum asiaticum var. japonicum*, a plant native to Jeju Island, South Korea, on the promotion of hair growth. Methods: We evaluated the promotion effects of the extract of *C. asiaticum* by organ culture method using a rat vibrissa follicles. To investigate the effect of the extract of *C. asiaticum* on anagen induction, the *C. asiaticum* extract was topical applied onto the back of C57BL/6 mice every day. To examine the proliferation of dermal papilla cells (DPCs), MTT assay was performed in immortalized rat vibrissa DPCs. Results: When rat vibrissa follicles were treated with extract of *C. asiaticum*, the hair-fiber lengths of the vibrissa follicles significantly increased. In addition, after topical application of the extract of *C. asiaticum* onto the back of C57BL/6 mice every day, anagen progression of the hair shaft was induced. When immortalized vibrissa DPCs were treated with extract of *C. asiaticum*, the extract increased the proliferation of DPCs. Conclusion: These results suggest that *C. asiaticum* extract stimulate hair growth in vitro and in vivo by the proliferation of DPCs. Acknowledgements. This work was supported by Brain Korea 21 program of the Korea Research Foundation.

753/B718

Identification of Premature Ovarian Failure-associated Gene, the Protein L-Isoaspartate (D-Aspartate) O-Methyltransferase.

J. Pyun, H. Kang, S. Lee, M. Kim, H. Kim, K. Kim, J. Kim, A. Oh, K. Lee, H. Song, S. Won, K. Kwack; CHA Research Institute, Pochon CHA Univ., Seongnam, South Korea

Premature ovarian failure (POF) is a condition arising amenorrhea before the age of 40 years. Primary amenorrhea is defined as the absence of a menarche and secondary amenorrhea is the interruption of ovarian function before the age of 40 years. Premature ovarian failure (POF) is a complex and heterogeneous disorder that is influenced by multiple genetic components. We performed two-stage association study to reveal some important genes associated with POF. At the first-stage association study, we found many regions which were associated with POF by performing a whole genome association study. One of the regions strongly associated with POF was on chromosome 6 and encoded protein L-isoaspartate (D-aspartate) O-methyltransferase (PCMT1). We identified that four polymorphisms in PCMT1 gene encoding a protein repair enzyme were associated with POF. All four polymorphisms formed a haplotype block, and all SNPs were in strong LD. We found four haplotypes and the frequencies of two haplotypes were statistically significantly different between each group. Haplotype 1 (ht1: AAGA) was highly distributed in the POF group, and ht2 (GGAT) was highly distributed in the matched control group. At the second-stage association study, we performed the disease association study for rs4816, which is a tagging SNP and also a nonsynonymous SNP, in a larger sample size. In the result, we found that the genotype frequencies of each group showed statistically significant differences. Our results suggest that ht1 is significantly associated with susceptibility to POF whereas ht2 is significantly associated with resistance to POF. In addition, the results of rs4816 obtained with a larger sample size showed rs4816 to be truly associated with POF.

754/B719

Neogenin Regulation of Iron Homeostasis.

D. Lee, Z. Zhou, L. Zhou, L. Mei, W. Xiong; Institute of Molecular Medicine and Genetics & Department of Neurology, Medical College of Georgia, Augusta, GA

Hemojuvelin (HJV), also called RGMc (Repulsive Guidance Molecule c) or HFE2, is a glycosylphosphatidylinositol (GPI)-linked cell surface protein that plays an important role in regulating iron homeostasis and is involved in the pathogenesis of human juvenile hemochromatosis. Neogenin, an immunoglobin super family transmembrane protein, is identified as a binding partner of HJV. However, the role of neogenin in iron homeostasis remains largely unknown. Here we present evidence for neogenin in this event. Neogenin deficient mice exhibit iron overload, decreased hepcidin expression, and reduced bone morphogenetic protein (BMP) signaling in liver, phenotypes resembling those observed in HJV mutant mice and supporting a role for neogenin in HJV-regulated iron homeostasis. In addition, we demonstrate that neogenin and HJV are co-expressed in peri-venous hepatocytes in developing mouse liver. Neogenin deficient liver/hepatocytes show altered distribution of HJV, decreased HJV protein level, and reduced BMP2 induced phosphorylation of Smad1/5/8. These results lead us to propose that neogenin may, via HJV, enhance BMP signaling and BMP-induced hepcidin expression in hepatocytes, thereby, regulating body iron homeostasis. These results also demonstrate a function of neogenin in iron homeostasis, and reveal a new mechanism underlying regulation of HJV signaling

Symposium III: Development and Regeneration

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Regeneration, Remodeling, and Renewal of the Intestine in the Planarian Schmidtea mediterranea.

D. Forsthoefel[1], D. Escobar[1], J. Stary[2], P. Newmark[1,2,3]; [1]Department of Cell & Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, IL, [2]Neuroscience Program, University of Illinois at Urbana-Champaign, Urbana, IL, [3]Howard Hughes Medical Institute, Urbana, IL

Freshwater planarians are capable of remarkable feats of regeneration in which tiny fragments of the body can give rise to complete animals. These regenerative abilities are based upon a stem cell population maintained throughout the animal's adult life. The stem cells, known as neoblasts, are the only proliferating somatic cells in the organism. In intact planarians, neoblasts serve as the source of new cells during physiological cell turnover; after amputation, they proliferate and differentiate to replace missing tissues. Numerous technical advances have led to a resurgence of interest in this classic model organism. In spite of recent progress in identifying genes required for stem cell function and proper regeneration, the dynamics of the renewal and regeneration of planarian organs remain poorly characterized at the cellular and molecular levels. The planarian intestine serves as a useful model for understanding how cell turnover is choreographed in tissues of uninjured animals and how complex organs are regenerated. We will describe the results of experiments to measure the amount of cell replacement in the intact and regenerating intestine and to determine the respective contributions of newly born intestinal cells and the remodeling of pre-existing intestinal tissue during the course of regeneration. We will also describe our ongoing efforts to decipher the gene expression programs underlying differentiation of the intestinal cells, to generate intestine-specific monoclonal antibodies, as well as to functionally characterize genes required for intestinal regeneration, remodeling, and homeostasis.

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Regulation and Regeneration during Endodermal Organ Formation in Zebrafish.

D. Stainier, D. Shin, S. Curado, R. Anderson, O. Andersson, D. Hesselson; Department of Biochemistry and Biophysics, University of California San Francisco, San Francisco, CA

Zebrafish embryos lacking Wnt2bb function initially fail to form a liver. Despite this initial defect, a liver eventually forms in a majority of the wnt2bb mutant embryos, which then develop into fertile adults. In order to gain insight into this recovery process, we have examined the role of Bmp and Fgf signaling, which are both required for liver formation. Surprisingly, over-activation of Fgf signaling in wnt2bb mutant embryos blocked the liver recovery process. We have also generated embryos with a more severe defect in Wnt signaling and these embryos exhibit a complete block in liver formation. However, blocking Fgf signaling in these embryos also rescued liver formation. We identified Fgf10 as the main Fgf responsible for this phenomenon, and used a chemical inhibitor of Fgf signaling to delineate the critical time-window. These and other data implicate the hepatopancreatic duct and its progenitors as a source of cells that allows the recovery of the liver in these experimental embryos. These gain- and loss-of-function experiments illustrate the importance of Fgf signaling and the hepatopancreatic duct in the zebrafish embryo's ability to regulate during endodermal organ development. In order to compare the process of regulation with that of regeneration, we have developed a system, based on the bacterial nitroreductase gene, to ablate specific lineages in zebrafish. We will present data pertaining to the regeneration of hepatocytes and pancreatic beta cells, trying to understand the cellular origin of the new cells as well as the signaling pathways that regulate their emergence.

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Epithelial Progenitor Cells in Lung Development and Repair.

B. Hogan; Department of Cell Biology, Duke University Medical Center, Durham, NC

The lung is a complex, three-dimensional organ made up of many different cell types. The most obvious is the tree-like system of endoderm-derived epithelial tubes and terminals airsacs (alveoli) through which air flows. However, lung function also critically depends on mesodermally-derived cells such as blood vessels, bronchial smooth muscle, lymphatics and myofibroblasts that develop in concert with the endodermal tubes. Another important cell population is the surface mesothelium (pleura). Unlike the situation in other endodermally-derived organs such as the stomach and intestine, epithelial cell turnover in the normal lung is very low. However, in models of epithelial cell damage, the lung demonstrates a remarkable capacity for repair. We have utilized a combination of in vivo lineage tracing studies, transgenic reporter lines, flow cytometry and in vitro culture to test competing ideas about the identity and potential of progenitor/stem cells of the lung during embryonic development, steady state maintenance and repair. Our data suggest that during embryonic development the different epithelial cells types are all derived from a population of multipotent progenitor cells at the tips of the growing tubes. During maintenance and repair, each region of the lung- the trachea, the airways, and the alveoli - depends upon a different population of progenitor/stem cells. Significantly, we find no evidence for a bipotential population of epithelial stem cells that contributes to both the airways and the alveoli. Finally, we have obtained evidence that during development mesothelial cells on the lung surface give rise to cells within the lung, including vascular smooth muscle.

Education Initiative Forum I: Learning Scientific Content with Research and Reflection on Life and Death Issues (758)

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Learning Scientific Content with Research and Reflection on Life and Death Issues.

K. K. Wentz-Hunter; Biological, Chemical and Physical Sciences, Roosevelt University, Chicago, IL

Cancer Biology [BIOL350/450] was a new course offered in the Spring 2008. In an effort to incorporate scientific content with social justice and creative writing, BIOL350/450 was designed with three major components. The first component provides students with knowledge of the

fundamental principles of molecular and cellular biology of cancer cells. Cancer Biology was designed to illustrate basic aspects of cancer development, and to discuss how molecular genetic approaches can be used to reveal fundamental processes of carcinogenesis. The second component involves civic engagement and social justice. The students in the class were required to be actively involved in the formation of an American Cancer Society Colleges Against Cancer [CAC] student organization chapter at Roosevelt University. The third component was an activity in creative writing as well as research. Students were "given" symptoms of particular cancers and asked to research their symptoms, type of cancer, and treatment regimens from a variety of sources. In addition, students made decisions on their treatment options taking into consideration their insurance status and personal situations. Students maintained journals of their activities and were graded for accuracy of research and completion of objectives. The desired learning outcomes included: comprehension of the cellular and molecular aspects of cancer progression; evaluation of cancer risks and treatments; involvement in the Roosevelt University student community for cancer education and advocacy through the CAC; and appreciation of what cancer patients experience upon diagnosis and subsequent treatment via creative writing exercises in a journal through reflections and decision making.

Symposium IV: Cell Migration and Metastasis (759 – 761)

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The Cell Biology of Metastasis: Imaging, Capture and Interrogation of Metastatic Breast Tumor Cells In Vivo.

J. Condeelis; Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, NY

The hallmark of metastatic cancer is the migration of tumor cells to distant sites in the body. The recent development of multi-photon based intravital imaging has revealed the behavior of individual tumor cells in breast tumors in vivo and these observations have inspired methods for the capture of these metastatic tumor cells from the primary tumor as a live cell population. Analysis of the properties of these live tumor cells has revealed that they are non-apoptotic, chemotherapy resistant and capable of chemotactic migration at velocities exceeding 10 times that of mesenchymal cells. Expression profiling of these live metastatic tumor cells has identified the genes involved in their survival, adjuvant-resistance and chemotaxis in vivo. These genes fall into well defined pathways which are coordinately regulated in tumor cells. This pattern is called the Invasion Signature and includes the cofilin/LIM-kinase, Mena/capping protein, N-WASP/Arp2-3 complex and ZBP1/mRNA pathways. Several pathways of the Invasion Signature are particularly relevant to the contribution of the migratory behavior of tumor cells to metastasis because 1) the pathways describe the molecular basis of chemotaxis to blood vessels used in the escape of cancer cells from primary breast tumors, and 2) these pathways contain makers that can be used to define the microenvironment of metastasis leading to intravasation and hematogenous dissemination of tumor cells. Key genes in the Invasion Signature have been studied for their ability to alter metastatic outcome and confirmed to be essential for metastasis in animal models using high resolution optical intravital imaging. In addition, the Invasion Signature provides several new biomarkers for predicting prognosis which have been used successfully in the clinic and these will be described.

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Bidirectional Leukocyte Chemotaxis in Zebrafish.

A. Huttenlocher[1,2], J. Mathias[1], M. Dodd[1], S. Yoo[1], K. Walters[1]; [1]Department of Medical Microbiology and Immunology, University of Wisconsin School of Medicine, Madison, WI, [2]Department of Pediatrics, University of Wisconsin, Madison, WI

The directional migration of cells within multicellular organisms is governed by competing gradients of both chemical attractants and repellents in diverse processes including embryonic development, leukocyte trafficking and axonal guidance. Neutrophil chemotaxis to sites of inflammation is a critical process during both normal immune responses to tissue injury and infection, and pathological conditions associated with chronic inflammation. To define the mechanisms that regulate neutrophil chemotaxis in vivo, we have developed novel transgenic zebrafish models in which the neutrophils express GFP or other probes under control of the myeloperoxidase promoter (zMPO:GFP). Tissue injury induces a robust, inflammatory response, which is characterized by the rapid chemotaxis of neutrophils to the wound site. In vivo time-lapse imaging shows that neutrophils subsequently display directed reverse chemotaxis back toward the vasculature to resolve the inflammatory response. Neutrophil reverse migration and resolution of inflammation is impaired in the context of some zebrafish models of chronic inflammation. Our findings implicate a novel mechanism involved in the resolution of the inflammatory response by the reverse chemotaxis of neutrophils back to the vasculature in zebrafish models of acute and chronic inflammation.

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Mechanisms of Metastasis.

J. Massagué; Cancer Biology and Genetics Program, Memorial Sloan-Kettering/HHMI, New York, NY

We are using complementary approaches including in vivo selection, gene expression profiling, clinical validation, and functional dissection, with the objective of identifying genes and microRNAs whose expression is clinically and functionally associated with metastasis to bones, lungs or brain. We identified and clinically validated microRNAs that suppress breast cancer metastasis by inhibiting tumor cell proliferation (miR-126) or inhibiting expression of the pro-invasive progenitor cell factors Sox4 and Tenascin-C (miR-335). We also identified organ-selective mediators of metastasis to lung, bone or brain. Eighteen genes whose expression in primary tumors is associated with pulmonary relapse in breast cancer patients constitute a lung metastasis signature (LMS). The LMS has minimal overlap with a bone metastasis gene set identified in the same source, the MDA-MB-231 ER-negative breast cancer cell line. Clinical and experimental evidence suggests that some LMS genes (ID1, ID3) support tumor re-initiation in the pulmonary parenchyma by tumor-propagating cells, whereas other genes (EREG, COX2, MMP1, Angiopoietin-like4) support tumor cell extravasation into the lung parenchyma. Mediators of breast cancer osteolytic metastasis identified in this manner include CXCR4, IL-11, CTGF, ADAMTS-1, follistatin and others. Applying this approach to the identification of genes and functions that mediate brain metastasis breast cancer cells and lung adenocarcinoma cells is shedding light on the identity of mediators of tumor cell extravasation across the blood-brain barrier. Using a complementary approach, we are determining the contribution of specific signaling pathways, such as the TGFβ pathway, to the genesis of organ-specific metastasis. Overlapping gene sets

identified with these two approaches are allowing us to prioritize metastasis mediators for detailed functional analysis. The identification of clinically relevant genes involved in basal and organ-selective metastasis is shedding light on the molecular and cellular mechanisms underlying these processes.

Minisymposium 9: Actin and Actin-related Proteins (762 – 767)

762

Non-sarcomeric Organization of Actomyosin II Filament Bundles in Polarizing Cells.

L. Cramer[1], T. Mseka[1], P. Coughlin[2]; [1]MRC Laboratory for Molecular Cell Biology (LMCB), University College London, London, United Kingdom, [2]Systems Biology, Harvard Medical School, Boston, MA

Actin reorganization is required for cell polarization and subsequent triggering of cell migration. Progress has been made in recent years on the identification of actin-binding proteins involved and their spatial and temporal regulation. However, not yet known for any polarizing cell, is how actomyosin II filament structures, which are major contributors within cells to the generation of motile-force driving cell polarization are organized. This prevents progress in elucidating precise molecular force-generating mechanisms involved. We have determined by electron microscopy and actin filament decoration, actin organization and filament polarity of actomyosin II filament bundles in primary chick fibroblasts that are destined to polarize and migrate. The revealed organization is distinct to muscle sarcomeres and actin stress fibres, thus identifying a non-sarcomeric mode of myosin II force-generation driving cell polarization in fibroblasts.

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Profilin 1 Is Required for Midbody Separation during Late Cytokinesis of Chondrocytes.

R. T. Boettcher, S. Wiesner, A. Braun, R. Faessler; Molecular Medicine, Max Planck Institute of Biochemistry, Martinsried, Germany

Profilins, the most versatile actin monomer-binding proteins, are abundantly expressed in all eukaryotic cells from Dictyostelium to mammals. Genetic and cell biological studies have implicated profilins in many cellular processes such as cell migration, cytokinesis, endocytosis and transcription regulation. However, the vital functions of profilins in differentiated mammalian cells are uncertain because complete profilin deficiency is lethal for early embryos of higher eukaryotes. To examine profilin function in chondrocytes we generated a cartilage-specific null allele of mouse profilin 1 (Col2pfn1). Homozygous Col2pfn1 mice develop progressive chondrodysplasia caused by disorganization of the growth plate and defective chondrocyte cytokinesis, indicated by the appearance of binucleated cells. Interestingly, we uncovered an important role for profilin in late cytokinesis instead of actomyosin ring formation. This is surprising as profilins are generally considered to be linked with formin activity in contractile ring formation. Col2pfn1 primary chondrocytes assemble and contract actomyosin rings during cell division, however they display defects during late cytokinesis as they frequently fail to complete abscission due to their inability to develop strong traction forces. This reduced force generation could be linked to abnormal cell spreading, focal adhesion assembly and stress fiber formation, which resulted from an impaired mDia1-mediated actin nucleation. Together, our results demonstrate that profilin 1 enables chondrocytes to generate sufficient abscission force for midbody separation in late cytokinesis.

764

p53-cofactor JMY Is a Multifunctional Actin Nucleation Factor.

J. Zuchero, D. Mullins; UCSF, San Francisco, CA

Polymerization of actin into filaments powers diverse cellular functions, and a key way that cells build distinct types of actin networks is by using different actin nucleation factors. These include the Arp2/3 complex, which creates branched filament networks, and Spire, which uses tandem repeats of an actin monomer-binding domain to create unbranched filaments. We find that JMY, a protein first identified as a vertebrate transcriptional co-activator of p53, combines these two nucleating activities by (1) activating the Arp2/3 complex and (2) assembling filaments directly by a Spire-like mechanism. Expression of JMY in mammalian cells induces polymerization of actin filaments in the cytoplasm. Consistent with a role in regulating transcription, endogenous JMY is nuclear in mammalian fibroblasts, and in undifferentiated HL-60 cells. However, when cells differentiate into a more motile phenotype, JMY moves from the nucleus to the cytoplasm, where it colocalizes with actin filaments at the leading edge. Altering cellular concentrations of JMY by overexpression or RNA interference leads to defects in cell migration, suggesting a role for JMY in motility. We propose a model in which nucleo-cytoplasmic shuttling of JMY controls its functions: regulating transcription in the nucleus of quiescent cells, and generating actin filaments to jump-start polymerization by the Arp2/3 complex in the cytoplasm of motile cells.

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Structure of the Actin-depolymerizing-factor Homology Domain in Complex with Actin.

V. O. Paavilainen[1,2], E. Oksanen[1], A. Goldman[1], P. Lappalainen[1]; [1]Institute of Biotechnology, University of Helsinki, Helsinki, Finland, [2]CMP, UC-San Francisco, San Francisco, CA

Actin filament turnover provides the driving force, required for cellular processes such as cell motility and endocytosis. Many proteins regulate the formation of new actin filaments or the polymerization kinetics of fast-growing actin filament ends. However, only one protein has been identified that accelerates the depolymerization of existing filaments, a key step of actin turnover in cells. This protein, ADF/cofilin, has been extensively characterized; it preferentially binds to actin filaments in their ADP-bound form, and this binding leads to the rapid depolymerization of these filaments. Despite the wealth of biochemical and cell biological information about ADF/cofilin, the molecular details of its interactions with actin have remained unclear. Like ADF/cofilin, the C-terminal ADF-H domain of twinfilin binds actin monomers and filaments and causes filament depolymerization, and is therefore a good model to study ADF/cofilin function. Here we present the x-ray crystal structure of the C-terminal ADF-H domain of twinfilin in complex with an actin monomer. This domain binds between actin subdomains 1 and 3 through an interface that is conserved in all ADF-H domain proteins. Based on the crystal structure, we propose a novel structural model for how ADF/cofilin interacts with actin filaments. This model suggests that ADF/cofilin binding to actin

filaments induces a conformational change in the actin protomers, and that these changes result in weakening of intrafilament interactions, and eventually depolymerization of the filaments.

766

WHAMM Is an Arp2/3-Activating Protein That Binds Microtubules and Functions in ER to Golgi Transport.

K. Campellone, N. Webb, E. Znameroski, M. Welch; Molecular and Cell Biology, University of California, Berkeley, CA

The Arp2/3 complex is an actin nucleator that plays a critical role in many essential cellular processes. Its activities are regulated by nucleation-promoting factors (NPFs), such as the WASPs (WASP and N-WASP) and the WAVEs (Scar/WAVE1-3), which function primarily during plasma membrane dynamics. Given the important roles of Arp2/3 activators in modulating actin assembly, we sought to identify additional NPFs and uncovered a mammalian protein that we named WHAMM (WASP homologue associated with actin, membranes, and microtubules). Unlike other NPFs, WHAMM localizes to the cis-Golgi apparatus and tubulo-vesicular membrane transport intermediates that lie along microtubules. Overexpression and depletion studies indicate that WHAMM is important for maintaining Golgi structure and facilitating ER to Golgi transport. The modular organization of WHAMM includes an N-terminal domain that mediates Golgi membrane association, a coiled-coil region that binds to microtubules, and a WCA segment that stimulates Arp2/3-mediated actin polymerization. Interestingly, the ability of WHAMM to interact with microtubules plays a role in membrane tubulation, while its capacity to induce actin assembly promotes tubule elongation. Overexpression of the small GTPase Rab1 stimulates both the formation and elongation of these tubules, suggesting that WHAMM and Rab1 cooperate to promote membrane tubulation during transport between the ER and Golgi. Thus, WHAMM is an important regulator of membrane dynamics that functions at the interface of the microtubule and actin cytoskeletons and may be modulated by Rab GTPases.

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Interplay between the Actin Cytoskeleton, Microtubules, and Cell Adhesion: Roles of Dia1 Formin.

A. Bershadsky[1], Y. Zilberman[1], L. Carramusa[1], T. Shemesh[3], M. Kozlov[3], c. ballestrem[2]; [1]Department of Molecular Cell Biology, Weizmann Institute of Science, Rehovot, Israel, [2]The Wellcome Trust Centre for Cell-Matrix Research, Faculty of Life Sciences University of Manchester, Manchester, United Kingdom, [3]Department of Physiology and Pharmacology, Sackler Faculty of Medicine Tel Aviv University, Tel Aviv, Israel

The formin homology proteins (formins) are potent activators of actin filament nucleation and elongation. We have previously proposed that formins may participate in the force-dependent regulation of the actin assembly. Here, we demonstrated that Dia1 formin, a target of RhoA, localizes to and controls the cadherin-mediated cell-cell adherens junctions. shRNA-mediated downregulation of Dia1 disrupted adherens junctions, as manifested by significantly decreased localization of E-cadherin and associated proteins to cell-cell contacts. Dia1 localized to the cell-cell junctions in a RhoA-dependent manner. This localization was accompanied by a strong increase in the width of the adhesion zone and augmentation of the actin, E-cadherin, and beta-catenin content in the junctions. Inhibition of myosin II activity abolished the Dia1-mediated reinforcement of cell-cell junctions. Formins are also involved in the actin-microtubule crosstalk. We have shown that knockdown of Dia1 triggers an increase in microtubule growth velocity, while constitutively active Dia1 slows it down. The effect of active Dia1 on microtubule dynamics was actin polymerization dependent. Looking for the molecular mechanisms underlying these dynamic alterations, we found that Dia1 can interact with a component of dynactin complex, actin related protein 1 (Arp1), a closest relative of actin. Dia1 inhibited the movement of Arp1-positive patches along microtubules in an actin dependent manner. Moreover, uncoupling Arp1 from microtubules by disruption of dynactin complex partially abolished the effect of Dia1 on microtubule dynamics. In search of physiological role of Dia1 in dynactin-mediated intracellular transport, we have shown that both RhoA and active Dia1 trigger in HeLa cells dispersion of Golgi complex in actin- and myosin-II-dependent manner, and depletion of the Dia1 abolished RhoA effect on Golgi. Microtubule stabilization by taxol greatly enhanced this Dia1 effect, indicating to involvement of microtubules in the Dia1-mediated Golgi dispersion. We hypothesize that Dia1 serves as a link between dynactin and the actin cytoskeleton.

Minisymposium 10: Cell Biology of the Synapse (768 – 773)

768

A Septin7-dependent Diffusion Barrier at the Neck of Dendritic Spines.

H. Ewers[1], T. Tada[2], M. Sheng[2], D. Choquet[1]; [1]CNRS UMR 5091, Université de Bordeaux2, Bordeaux, France, [2]The Picower Institute for Learning and Memory RIKEN-MIT Neuroscience Research Center Howard Hughes Medical Institute, Massachusetts Institute of Technology, Cambridge, MA

Septins are cytoskeletal GTPases that form heteromeric hexamers and can assemble into higher molecular order complexes. In neuronal cells, septin complexes are located to dendritic branching points during development and later to the neck of dendritic spines, where they control dendritic spine morphology. However, the concrete functional role of the septin complex at the spine neck is unclear. Here, we have investigated a possible role of Septin7 as part of a diffusion barrier at the neck of dendritic spines in cultured hippocampal neurons. We studied the stability of the Septin7 complex at the base of dendritic spines and its influence on membrane protein mobility into and out-of dendritic spine membrane. Fluorescence time-lapse imaging and FRAP of Septin7-GFP showed that Septin7-GFP is immobile at the spine neck and forms a complex that is stable for more than 30min. By single particle tracking of endogenous glutamate receptors via quantum-dots we found that receptor diffusion is slower in Septin7 positive regions, especially at the spine neck. Furthermore, receptors showed a prolonged dwell-time on Septin7 positive spines in respect to spines that did not bear Septin7 staining at their neck. A fluorescence-tagged transmembrane protein and a fluorescent protein targeted to the inner membrane leaflet both showed reduced recovery rates after photobleaching in Septin7 positive spines compared to Septin7 negative spines. These data are consistent with the idea that septin7 is part of a stable complex at the spine neck that forms a barrier to lateral diffusion from the parent dendrite into the spine plasma membrane and vice-versa.

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Architecture of the Actin Cytoskeleton in Dendritic Spines and Dendritic Filopodia.

F. Korobova, T. Svitkina; Department of Biology, University of Pennsylvania, Philadelphia, PA

Dendritic spines are tiny protrusions on the surface of dendrites that make contacts with axons and form excitatory synapses in the brain. The morphological plasticity of spines plays a pivotal role in learning and memory, and is heavily based on dynamics of the actin cytoskeleton. However, the actin cytoskeleton architecture in dendritic spines and their precursors, dendritic filopodia, is poorly understood. We used platinum replica electron microscopy to investigate the actin organization in dendritic spines and filopodia in cultured hippocampal neurons. The mushroom-shaped spines consisted of a bulbous head, a constricted stalk, and a delta-shaped base. In thin and stubby spines, stalks were longer or absent, respectively, while heads in thin spines might be relatively small. Spine heads contained a dense meshwork of short and branched actin filaments and they were immunopositive for Arp2/3 complex and capping protein. Stalks contained a variable number of bundled or loosely arranged longitudinal filaments. The spine bases were typically formed by long actin filaments coming from the dendrite and converging at the beginning of the stalk, but might also contain branched networks and the Arp2/3 complex signal. Polarity determination revealed that actin filament barbed ends in all compartments were mostly oriented away from the dendrite, although the opposite orientation sometimes was seen in the stalk and the base. Actin-binding protein drebrin was found throughout the spine except for the very distal region of the head, whereas myosin II was found in spine bases and stalks. Dendritic filopodia in young neurons showed strikingly different actin organization, as compared to the growth cone filopodia, and instead of tight bundles of long filaments, they contained loosely aligned actin filaments of varying length with occasional patches of branched networks at the side and/or the tip of the filopodium. Moreover, unlike growth cone filopodia, myosin II, drebrin and capping protein were present in dendritic filopodia. Thus, we found that, within a small volume, dendritic spines and dendritic filopodia appear to combine both protrusive and contractile actin-dependent machineries. Supported by NIH grant GM 070898.

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Reversible Binding to F-Actin by CPG2, a Regulator of Glutamate Receptor Endocytosis.

S. Loebrich[1], J. Gibson[1], E. Nedivi[2,1]; [1]Picower Institute for Learning and Memory, Massachusetts Institute of Technology, Cambridge, MA, [2]Department of Biology, Massachusetts Institute of Technology, Cambridge, MA

The contribution of the F-actin cytoskeleton to clathrin-mediated endocytosis in mammals is only beginning to be understood. Recent studies show that coupling of cargo to F-actin alters internalization kinetics. Removal or insertion of AMPA-type glutamate receptors has been proposed as a mechanism for regulating the strength of synaptic connections in the brain. Glutamate receptor endocytosis is clathrin-mediated and takes place in an endocytic zone within the dendritic spine. Candidate Plasticity Gene 2 (cpg2), identified in a screen for activity-regulated genes, encodes a protein that localizes exclusively to the endocytic zone in spine heads. CPG2 knockdown impairs both constitutive and agonist-induced internalization of AMPA receptors, and leads to accumulation of clathrin-coated vesicles in the postsynaptic compartment, suggesting that CPG2 is required for uncoating or recycling of endocytic vesicles. Here, we present evidence that CPG2 interacts with a set of proteins known to be cytoskeletal associated, and that in cultured neurons CPG2 is physically linked to F-actin filaments within mature spines and cannot be removed by cold Triton extraction. Using an in vitro binding assay, we mapped the F-actin binding region in CPG2 to two predicted coiled-coil domains in the C-terminus. These two coiled-coils together are sufficient to confer F-actin binding activity onto an unrelated protein sequence and constitute a novel, bipartite F-actin binding domain. We further mapped a 100 amino acid stretch of CPG2 upstream of the F-actin binding motif that represses F-actin binding by the coiled coil domains. Since CPG2 is a predicted target of PKA, PKC and CamKII phosphorylation, we are currently testing whether introducing site-specific phosphomimetic mutations within the repressor region negates repressor function. We speculate that CPG2 enables glutamate receptor endocytosis by anchoring elements of the endocytic machinery to F-actin in the spine cytoskeleton, and that CPG2 phosphorylation in the repressor domain may regulate this interaction with F-actin.

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Role of the Phosphoinositide Metabolism in Synaptic Transmission and Plasticity in Hippocampal Neurons.

S. Jurado[1], K. L. Arendt[1], C. Petrok[1], J. Esteban[2,1]; [1]Department of Pharmacology, University of Michigan, Ann Arbor, MI, [2]Centro de Biología Molecular "Severo Ochoa", Universidad Autónoma de Madrid (UAM), Madrid, Spain

The turnover of the phosphoinositide PI(3,4,5)P3 is tightly controlled by the tumor suppressor PTEN (Phosphatase and Tensin homolog deleted in chromosome Ten). PTEN is a lipid phosphatase that catalyzes the conversion of PI(3,4,5)P3 into PI(4,5)P2. The role of PTEN in controlling cell proliferation during development is well established. However, very little is known of a potential function of PTEN in the mature nervous system. In the present study we have investigated the role of PTEN in PIP3 turnover in neurons, and its involvement in the regulation of synaptic function. In particular, we have found that enhancement of PTEN function leads to depression of synaptic transmission in hippocampal neurons. In addition, we demonstrate that PTEN catalytic activity is required for a form of synaptic plasticity known as long-term depression (LTD). PTEN is known to interact with PDZ domain-containing proteins, such as MAGI-1/2/3. Interestingly, we have found that PTEN interaction with PDZ proteins is regulated by neuronal activity, and controls its recruitment to synaptic sites during LTD. In summary these results uncover a new role of PTEN in the regulation of synaptic transmission and plasticity in hippocampal excitatory synapses.

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Regulation of Synapse Formation by p120 Catenin and Fer Tyrosine Kinase.

S. Lee[1], I. Peng[1], S. Ng[1], M. Yanagisawa[2], B. X. Shernaz[4], L. Elia[5], J. Balsamo[3], J. Lilien[3], P. Anastasiadis[2], E. Ullian[1], L. F. Reichardt[1]; [1]UCSF, San Francisco, CA, [2]Mayo Clinic, Jacksonville, FL, [3]University of Iowa, Iowa City, IA, [4]University of British Columbia, Vancouver, BC, Canada, [5]Elan Pharmaceuticals, South San Francisco, CA

Studies on the mechanisms of synapse development and function have indicated the importance of cell adhesion molecules. Cadherins are calcium-dependent intercellular adhesion molecules that have been proposed to play important roles in synaptic development in association

with catenins. Recently beta-catenin has been shown to function as a scaffold protein to recruit and anchor PDZ domain-containing proteins to cadherin clusters. These scaffolds in turn localize synaptic vesicles at nascent synapses. Here we report roles for the nonreceptor tyrosine kinase Fer and p120 catenin in presynaptic development. Perturbation of Fer or p120 catenin function at the presynaptic compartment prevented normal presynaptic organization and excitatory synapse formation and destabilized synaptic vesicles and CAZ proteins. Knockdown of Fer or p120 catenin diminished presynaptic physiological function significantly. Fer function on presynaptic organization was dependent to its binding to p120 catenin. The effects of Fer appeared to be mediated through control of phosphorylation of beta-catenin. Our results also indicated that perturbations of Fer and p120 catenin have differential effects on presynaptic organization. Taken together, Fer in the presynaptic compartment mainly contributes to maintenance of the scaffold function of beta-catenin by transmitting signals leading to dephosphorylation of beta-catenin. Presynaptic p120 catenin may have essential roles in presynaptic development in addition to recruitment of Fer, such as control of cadherin stability or interaction and/or cytoskeletal organization in the presynaptic compartment.

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C. elegans Long-Term Associative Olfactory Memory Requires CREB Transcriptional Target Activity and JNK Signaling in Interneurons and Glia.

A. L. Kauffman, C. Murphy; Department of Molecular Biology, Princeton University, Princeton, NJ

C. elegans can associate chemical odors with nutritional and environmental conditions. However, the memory of these associations and the molecular requirements of associative memory are not known. We have developed two positive associative olfactory memory assays, and found that long-term, but not short-term, associative memory is dependent on *crh-1*, the *C. elegans* homolog of the CREB transcription factor. Using microarrays, we identified the CREB transcriptional targets that are changed during long-term memory training; this set includes genes involved in vesicle trafficking, synaptogenesis, RNA binding, neural migration, and serotonin signaling, as well as ion channels and uncharacterized genes. Many of these targets, including the CREB-regulated upstream activators of CREB, are required for long-term memory. LTAM training induces CREB-reporter GFP expression in the SIA and AIY interneurons, a pattern that differs from CREB activation during starvation. Additionally, downstream targets of CREB are activated in other neurons, including glia, likely through CREB's activation of the JNK pathway. Our data suggest that while the AWC mediates butanone sensing and learning, CREB-dependent long-term associative memory involves the activity of neighboring interneurons and a novel, active role for glial cells. Our findings provide a molecular and cellular framework to understand associative memory.

Minisymposium 12: Epigenetic Regulation (774 – 779)

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Epigenetic Genome Control by RNAi and Transposon-derived Proteins.

S. Grewal; CCR- Laboratory of Biochemistry and Molecular Biology, National Cancer Institute, Bethesda, MD

Eukaryotic genomes are often replete with transposable elements (TEs) and their remnants. These elements referred to as "junk DNA" have driven the genome evolution in diverse ways. Due to their proliferation and mutagenic potential, host genomes have evolved defense mechanisms against TEs including histone methylation and RNAi. However, these mechanisms have only minor roles in regulating Tf2 long terminal repeats (LTRs) retrotransposons in the fission yeast Schizosaccharomyces pombe. We have uncovered a novel genome surveillance mechanism for retrotransposons by a family of transposase-derived CENP-B homologs. S. pombe CENP-Bs localize at and recruit class I and II histone deacetylases to silence Tf2 retrotransposons. CENP-Bs also repress retrotransposon relics scattered throughout the S. pombe genome, and often located near gene promoters to exert influence on the expression of genes. Surprisingly, Tf2 elements dispersed throughout the genome are clustered into "Tf" bodies, the organization of which depends on CENP-Bs that display network-like nuclear structures. CENP-B-mediated surveillance is proactive, capable of preventing an "extinct" Tf1 retrotransposon from reentering the host genome by blocking its homologous recombination with extant Tf2, and silences and immobilizes a Tf1 integrant that, remarkably, becomes sequestered into Tf bodies. These results reveal a likely ancient retrotransposon surveillance pathway important for host genome organization and maintenance of genomic integrity. Our recent progress in understanding the mechanisms of epigenetic genome control by RNAi and transposon-derived CENP-B proteins will be discussed. Grewal, S.I.S. and Jia, S. (2007). Heterochromatin revisited. Nat. Rev. Genet., 8:35-46. Cam, H., Noma, K., Ebina, H., Levin, H. and Grewal S.I.S. (2008) Host genome surveillance for retrotransposons by transposon-derived proteins. Nature, 451: 431-436. Chen, E.S., Zhang, K., Nicolas, E., Cam, H., Zofall, M., and Grewal, S.I.S. (2008) Cell cycle control of centromeric repeat transcription and heterochromatin assembly. Nature, 451: 734-747.

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Reprogramming Towards Pluripotency in Heterokaryons Is Rapid and Efficient.

N. Bhutani[1], Y. Liao[1], A. Palermo[1], R. Doyonnas[1], Z. Ouyang[2], W. H. Wong[3,4], H. M. Blau[1]; [1]Department of Microbiology & Immunology, Stanford University, Stanford, CA, [2]Department of Biology, Stanford University, Stanford, CA, [3]Department of Statistics, Stanford University, Stanford, CA, [4]Department of Health Research and Policy, Stanford University, Stanford, CA

The generation of pluripotent cells from a patient's own somatic cells is the holy grail of regenerative medicine. A variety of techniques have been used to address this need including 'reprogramming' by transfer of somatic nuclei into oocytes (SCNT), by fusion of somatic cells with embryonic stem cells (ES), and introduction of defined factors into adult skin fibroblasts to generate induced-pluripotent stem cells (iPS). The iPS cells are very similar to ES cells in global gene expression, epigenetic status and their ability to generate all the germ layers. However, the frequency and efficiency of iPS clones generated from transduced cells is extremely low (0.1-1%) and the reprogramming is very slow (12-15 days post-transduction). We therefore hypothesize that additional factors are required to increase the efficiency of reprogramming. In order to identify key regulators of reprogramming, we have optimized a novel heterokaryon platform in which reprogramming of a somatic fibroblast occurs in the presence of the complete ES proteome. Human fibroblasts are fused with mouse ES cells using a chemical fusogen, PEG, to form stable, non-dividing heterokaryons in which the nuclei do not fuse and remain distinct. Taking advantage of the species

differences between human and mouse genomes, we have monitored global gene expression and epigenetic changes in the human nuclei in a sequential fashion within hours of fusion. The core ES regulators (Oct4, Sox2 and Nanog) are upregulated within 24-48 hours as compared to 18-20 days in the iPS, accompanied by rapid DNA demethylation at their promoters. Using a novel method for distinguishing mouse and human transcripts, Species Specific Transcriptome Amplification (SSTA), we have determined that nuclear reprogramming toward an ES cell state is global in heterokaryons. This method and the speed and efficiency of reprogramming in the heterokaryon system allow characterization of early critical factors and epigenetic regulators, providing insight into the mechanisms of reprogramming and enabling identification of novel candidate factors that can facilitate efficient derivation of iPS cells.

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Epigenetic Regulation of Gene Expression by the Drosophila Myb Oncoprotein and E2F2-RBF Tumor Suppressor Proteins.

H. Wen, L. Andrejka, J. Lipsick; Pathology & Genetics, Stanford University, Stanford, CA

The Drosophila Myb oncoprotein, the E2F2 transcriptional repressor, and the RBF and Mip130/LIN-9 tumor suppressor proteins reside in a conserved Myb-MuvB (MMB)/dREAM complex. We now show that Myb is required in vivo for the expression of Polo kinase and components of the spindle assembly checkpoint (SAC). Surprisingly, the highly conserved DNA-binding domain was not essential for assembly of Myb into MMB/dREAM, for transcriptional regulation in vivo, or for rescue of Myb null mutants to adult viability. E2F2, RBF, and Mip130/LIN-9 acted in opposition to Myb by repressing the expression of Polo and SAC genes in vivo. Remarkably, the absence of both Myb and Mip130, or of both Myb and E2F2, caused variegated expression in which high or low levels of Polo were stably inherited through successive cell divisions in imaginal wing discs. Restoration of Myb resulted in a uniformly high level of Polo expression similar to that seen in wild type tissue, whereas restoration of Mip130 or E2F2 extinguished Polo expression. Our results demonstrate epigenetic regulation of gene expression by Myb, Mip130/LIN-9, and E2F2-RBF in vivo, and also provide an explanation for the ability of Mip130 null mutants to rescue the lethality of Myb null mutants in vivo. Ref: Wen, et al, Genes & Development 22: 601-614 (2008).

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X-Chromosome-wide Repression.

B. Meyer; Department of Molecular and Cell Biology, University of California, Berkeley/HHMI, Berkeley, CA

Gene expression in metazoans is regulated not only at the level of individual genes but also in a coordinated manner across large chromosomal domains, such as telomeres or imprinted gene clusters, and along entire chromosomes, as in X-chromosome dosage compensation. Dosage compensation ensures that males (XO or XY) and females (XX) express equal levels of X-linked gene products, despite their difference in X-chromosome dose. In C. elegans, a specialized dosage compensation complex (DCC) is targeted to both X chromosomes of hermaphrodites to repress transcript levels by half. The DCC resembles condensin, a protein complex required for the compaction, resolution, and segregation of chromosomes during both mitosis and meiosis. We have shown the DCC not only resembles condensin, it shares a component with condensin, and DCC components also participate in other aspects of chromosome segregation, including the regulation of crossover control during meiosis. The DCC proteins in conjunction with condensin subunits regulate the number and distribution of crossovers by controlling double-strand break formation through changes in chromosome structure. Our work has revealed much of the mechanism by which the DCC recognizes and binds X chromosomes. DCC targeting to X involves its initial recruitment to specific sites called rex sites that are distributed along X to attract the DCC and to nucleate spreading to X sites lacking recruitment ability (dox sites). rex sites contain degenerate motifs important for DCC binding. Rex and dox sites are interspersed and separated by long distances, suggesting a spreading mechanism mediated by long-range interactions. The similarity of the DCC to condensin suggests that DCC spreading along X occurs through changes in chromosome structure. X targeting also involves protein modifications that deflect the DCC from sites on autosomes. Studies investigating the relationship between DCC binding location and its impact on the expression of neighboring genes show that the DCC does not act locally on a gene-by-gene basis but rather globally across large chromosomal territories.

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Mitotic Condensins Are Required for RNAi and Chromatin-based Gene Silencing.

J. Carey[1], T. Tabuchi[1], J. Thompson[2], J. R. Yates[2], K. Hagstrom[1]; [1]Program in Molecular Medicine & Program in Cell Dynamics, University of Massachusetts Medical School, Worcester, MA, [2]Department of Chemical Physiology, Scripps Research Institute, La Jolla, CA

Condensin proteins organize chromosome structure and facilitate chromosome condensation and segregation during mitosis. Here we describe a new role for condensins in RNA interference and chromatin-based gene silencing. Higher eukaryotes have two distinct mitotic condensin complexes: condensin I and condensin II. *C. elegans* was considered an exception because its condensin I-like complex (condensin I^{DC}) had no mitotic function. Instead, condensin I^{DC} served to dampen gene expression on hermaphrodite X chromosomes during dosage compensation. We resolved this discrepancy by identifying an additional condensin I complex (condensin I^{M}) that differs from condensin I^{DC} by a single subunit, yet binds to mitotic and meiotic chromosomes and promotes their segregation. Because mitotic condensins are structurally related to the X silencing complex, we wondered whether they might also have gene repression activity. In this light, we were intrigued to discover RNAi machinery and chromatin modifiers in immuoprecipitations of a condensin II subunit. We demonstrated that condensin subunits are required for repetitive transgene silencing, a phenomenon mediated by RNAi and histone modifying factors. Remarkably, mutations in condensin II subunits prevented robust gene silencing by exogenous dsRNA, suggesting that condensin II is required for the process of RNAi. These studies suggest a novel link between condensin control of mitotic chromosome structure and chromatin- and RNAi- mediated transcriptional gene silencing during development. We speculate that condensins could have a role in small RNA mediated formation of epigenetically heritable chromatin states.

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Nuclear Lamina Component Barrier-to-Autointegration Factor Interacts with Nucleosomes, Influences Histone Posttranslational Modifications In Vivo, and Has Physiological Roles in DNA Damage Response.

R. Montes de Oca[1], M. Resch[3], C. J. Shoemaker[1], M. Gucek[2], R. N. Cole[2], K. Luger[3], K. L. Wilson[1]; [1]Cell Biology, Johns Hopkins Medical

School, Baltimore, MD, [2]Mass Spectrometry/Proteomics Facility, Johns Hopkins Medical School, Baltimore, MD, [3]Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO

Barrier-to-autointegration factor (BAF) is a small, mobile and essential component of the nuclear lamina network. BAF binds directly to lamins (both A- and B-type), to nuclear LEM-domain proteins (LAP2, Emerin, MAN1), and core histones H3 and H4. BAF can interact with at least one class of transcription factor (Crx and related homeodomain transcription activators) and can regulate gene expression in specific tissues, including muscle. BAF also has structural roles in collaboration with lamins and LEM-domain proteins: BAF is required during mitosis for chromosome segregation and nuclear reassembly and is necessary during interphase for the integrity of higher-order chromatin organization. We found that nucleosomes are a physiological target for BAF. BAF can bind reconstituted mononucleosomes in vitro, co-purifies with mononucleosomes isolated from cells, and is co-immunoprecipitated by antibodies that recognize specific posttranslational modifications on histones H3 or H4. In cells that overexpressed BAF, the levels of several histone posttranslational modifications (acetylation, methylation, phosphorylation) were altered. To investigate the mechanism by which BAF altered the "epigenome", BAF-interacting proteins were affinity-purified from HeLa cell nuclear lysates and identified by LC-MS/MS and iTRAQ. We recovered several known interactors plus novel transcription factors, disease-relevant proteins, several enzymes involved in chromatin remodeling and silencing, and unexpectedly also proteins involved in DNA repair. Several tested candidates were independently validated in vivo. Our findings suggest BAF can influence gene expression and other chromatin activities by recruiting specific histone-modifying enzymes to chromatin. In addition, BAF interacted with components of the CUL4-DDB-ROC1 complex, which ubiquitylates histones at sites of DNA damage, in a time-dependent manner after UV-induced damage. Our results implicate BAF in both the epigenetic regulation of chromatin, and DNA repair, and suggest potential mechanisms for the dependence of chromatin regulation and repair on the lamina network.

Minisymposium 13: Imaging and Biosensors (780 – 785)

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Observing and Manipulating Proteins in Living Cells through Chemical Labeling.

K. Johnsson; Institut des Sciences et Ingénierie Chimiques, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

The visualization and characterization of biologically relevant molecules and activities inside living cells continues to transform cell biology into a truly quantitative science. However, despite the spectacular achievements in some areas of cell biology, the majority of cellular processes still operate invisibly. Further progress will therefore not only depend on improvements in instrumentation but increasingly on the development of new (fluorescent) sensors and other synthetic probes to target and characterize these activities. In this talk I will discuss how the specific labeling of proteins with chemically probes in vivo and in vitro can be used to study and to manipulate protein function as well as to create sensors for biochemical activities. Specifically, I will discuss a strategy for the simultaneous detection of multiple protein-protein interactions in mammalian cells based on a covalent cross-linking of two interacting partners. This approach makes use of cell-permeable crosslinkers that incorporate a fluorophore and two separate reactive groups that each reacts specifically with a self-labeling protein tag. Protein-protein interactions can be studied by fusing the proteins of interest to the two specific tags. Furthermore, we will discuss the labeling of fusion proteins with environmentally sensitive fluorophores and the construction of FRET-based nanosensors composed out of self-labeling proteins and autofluorescent proteins.

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New Tools to Visualize and Manipulate Dynamic Signaling Pathways In Vivo.

K. Hahn[1], A. Gulyani[1], A. Karginov[1], L. Hodgson[1], E. Vitriol[1], C. Welch[1], Y. Wu[1], R. Jha[4], M. Machacek[3], G. Danuser[3], B. Kay[2], B. Kuhlman[4]; [1]Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]Biology, University of Illinois, Chicago, IL, [3]Cell Biology, Scripps Research Institute, La Jolla, CA, [4]Biochemistry, University of North Carolina at Chapel Hill, Chapel Hill, NC

To better understand signals underlying rapid and dynamic cellular events, i.e. cell motility and cytoskeletal dynamics, we have been designing novel tools for both the visualization and manipulation of protein behavior in living cells. For protein manipulation with subsecond and microns resolution we have developed genetically encoded caged proteins, undergoing reversible activation upon irradiation with light of 450-500 nm. Caged Rac has revealed divergent roles for Pak, ROCK and MLCK signaling during Rac-induced motility. A general strategy for drug-induced kinase activation will be described, applied to decipher the role of FAK in cell protrusion and adhesion dynamics. New biosensors based upon FRET 'antenna systems' can be used to visualize endogenous Pak, Src and Cdc42 activation with genetically encoded sensors. Dye-based and FRET-based biosensors from the above studies are used in the same cell for high resolution studies of Rho GTPase coordination during cell motility.

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Genetically Encoded Sensors Illuminate Cellular Distribution of Zinc.

A. E. Palmer, P. J. Dittmer; Chemistry & Biochemistry, University of Colorado, Boulder, CO

Our current understanding of cellular metal homeostasis is limited by the lack of appropriate tools to interrogate metal ion availability and distribution within living cells. Many of the most basic questions remain unanswered because existing probes are incapable of providing insight into the spatial variability and localization of specific metal ions. As the second most abundant transition metal in biological organisms, Zn2+ represents an important target for improved sensors. Zn2+ is unique among transition metal ions, as it is concentrated into secretory vesicles within a number of mammalian tissues (brain, prostate, and pancreas). While the physiological role of vesicular Zn2+ is poorly defined, there is compelling evidence that it may play an active role in transmission of cellular signals (Frederickson 2005), representing an exciting new model for how transition metals influence cellular functions. Moreover there is emerging evidence that Zn2+ can be mobilized from bound stores suggesting that transient zinc signals are generated within cells. We have developed genetically encoded FRET-based sensors for Zn2+. These are the first genetically encoded sensors demonstrated to monitor Zn2+ distribution in live cells. We establish methodology to calibrate the probes in situ and demonstrate that the in situ Zn2+ affinities are the same as those measured in vitro.

In order to ultimately identify both the sources and sinks for Zn2+, we target these sensors to specific locations within the cell (namely cytoplasm, plasma membrane, and mitochondria). These localized probes reveal heterogeneity in the spatial distribution of Zn2+ under resting conditions. A distinct advantage of genetically encoded probes is the ability to define and vary the sensor concentration. In this work, we measure the resting Zn2+ concentrations as a function of the sensor protein concentration to define cellular Zn2+ buffering. Lastly, we express the mitochondrial-targeted sensor in hippocampal neurons and observe transient zinc signals in mitochondria following glutamate stimulation.

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Photoactivatable mCherry for the Two-Color High-Resolution Fluorescence Microscopy.

F. Subach[1], G. Patterson[2], S. Manley[2], J. Gillette[2], J. Lippincott-Schwartz[2], V. Verkhusha[1]; [1]Department of Anatomy and Structural Biology, and Biophotonics Center, Albert Einstein College of Medicine, Bronx, NY, [2]Section on Organelle Biology, Cell Biology and Metabolism Program, National Institute of Child Health and Human Development, National Institutes of Health, Bethesda, MD

Photoactivatable fluorescent proteins (PAFP) have found numerous applications in cell biology, but notably absent has been a dark-to-red PAFP of useful brightness and contrast. We have developed three dark-to-red photoactivatable variants of the red fluorescent protein mCherry. The best variant, called PAmCherry1, is originally dark and not detectable in any fluorescence emission filter but when is irradiated with a violet light, such as 405 nm laser line, it becomes a red fluorescent with the excitation/emission maxima at 564/595 nm. The photoactivation contrast of PAmCherry1 is 4,000-fold in purified protein samples and more than 100-fold in mammalian cells. The resulting fluorescence brightness is a half of that of mCherry. It also maintains the similar monomeric properties and the fast maturation rate as the parental protein. As compared to other monomeric or tandem-dimeric red PAFPs, PAmCherry1 has the higher pH stability with an apparent pKa of 6.5, the higher photostability, and the similar or higher number of photons per molecule in single-molecule experiments. It makes PAmCherry1 an excellent probe for both conventional diffraction-limited fluorescence microscopy and super-resolution imaging techniques such as a photoactivated localization microscopy (PALM). Moreover, lack of the background green fluorescence allows for a two-color PALM with green PAFPs. Two-color PALM imaging of PAmCherry1 tagged to the plasma membrane transferrin receptor (TfR) and PAGFP fused with the clathrin light chain (CLC) were performed using low level 405 nm laser for simultaneous PAmCherry1 and PAGFP photoactivation followed by sequential collection of 561 nm excited PAmCherry1 and 488 nm excited PAGFP single-molecule fluorescence. Pair correlation analysis of the PALM images suggests numerous clusters of less than 200 nm in size with both distinct and overlapping distributions of the TfR and CLC chimeras at sub-diffraction 25 nm resolutions. These different TfR and CLC clusters possibly represent sequential stages of clathrin-coated pit dynamics with and without the cargo. Since PAmCherry1 and PAGFP chimeras can be imaged nearly simultaneously, it opens the possibility of performing two-color PALM experiments in live cells.

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Single-Cell-Pico-Force-Microscopy: A Novel Tool for High Resolution Measurements of Cell Forces.

B. W. Axelrod[1], M. L. Roukes[1,2,3]; [1]Applied Physics, California Institute of Technology, Pasadena, CA, [2]Physics, California Institute of Technology, Pasadena, CA, [3]Bioengineering, California Institute of Technology, Pasadena, CA

We have developed a tool, which we call Single-Cell-Pico-Force-Microscopy (SCPFM), capable of measuring cellular forces from individual adherent cells with the high resolution necessary to track macro-molecular force dynamics in living cells. Existing tools capable of measuring cell forces, while varied, are all based on optical detection and are limited in sensitivity, range or the physiological relevance of their coupling to the cytoskeleton. SCPFM overcomes these limitations by utilizing novel polymer Nano-Electro-Mechanical-Systems (NEMS) force sensors. SCPFM is a fully integrated, monolithic instrument that combines the near-single molecule force measurement capabilities of (NEMS) based force sensors with integrated microfluidics that enable precision fluid control, and thereby precision pharmacological stimulation of individual cells. SCPFM enables for the first time the systematic study of the molecular-mechanical responses of individual cells to pharmacological and mechanical stimulation. Our initial results from studies of contracting and relaxing lamellipodia of pharmacologically perturbed cells will be presented. Forces exerted by individual adherent cells are measured with force resolution of 200pN and time resolution of 100ms. We observe small force oscillations, roughly 400pN peak-to-peak, with frequency that is monotonically dependent on the force being exerted by the lamellipodia. Additionally, large, stable, quantized force steps of order 1nN are manifested when a cell's cytoskeleton is perturbed with Cytochalasin D and allowed to recover in growth media. This work illustrates the immense potential of NEMS based tools for significantly enhanced resolution of cell biology at the single cell level.

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Determining Spatial and Temporal Limits of the Tumor Intravasation Microenvironment In Vivo.

B. Gligorijevic[1,2], D. Kedrin[1], J. Wyckoff[1,2], J. Segall[1], J. van Rheenen[1,2], J. Condeelis[1,2]; [1]Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, New York, NY, [2]Gruss Lipper Biophotonics Center, Albert Einstein College of Medicine, New York, NY

Most studies of cell motility, including assays which model invasion and intravasation processes, are done in in vitro culture models used cells grown on artificial matrices. Although a lot can be learned from these models, the results are often not directly applicable to complex tumor microenvironments as found in vivo. The objective of this study is to determine spatial and temporal borders of the tumor cell intravasation microenvironment in vivo, as the first step in describing tumor cell-stromal cell interactions essential for intravasation. We report a technique for monitoring the same tumor microenvironment over multiple imaging sessions in living animals. Individual tumor cells expressing photoswitchable protein Dendra2 in an orthotopic mammary carcinoma can be photo-converted from green to red and followed for extended times through an imaging window implanted on top of the mammary gland. We have followed two populations of tumor cells, one of which is positioned next to a flowing blood vessel, while the other one is more than five cell diameters away. One day (24h) after photoswitching, cells near the blood vessel orient around it, and their number is lower compared to the initial value. In contrast, cells far from the blood vessel show very little migration and a small increase in cell number, possibly due to cell replication. These results indicate that two distinct microenvironments in the same tumor affect the invasion and intravasation differently. The local tumor microenvironment that

supports invasion and intravasation of the primary tumor into the vasculature is composed of vascular structures and stroma that includes fibroblasts, immune cells and extracellular matrix. Investigating components of such microenvironments and interactions within them will improve our understanding of cancer progression and ultimately assist us in creating therapies based on disruption of the tumor-host interactions.

Minisymposium 14: Mitosis and Meiosis (786 – 791)

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Centromere Stretching and Mitotic Checkpoint.

C. B. O"Connell[1], J. Loncarek[1], P. Hergert[1], A. Kourtidis[2], D. Conklin[2], A. Khodjakov[1]; [1]New York State Department of Health, Wadsworth Center, Albany, NY, [2]GenNYSis, University at Albany, Rensselaer, NY

The accuracy of chromosome segregation during mitosis is enhanced by the spindle assembly checkpoint (SAC). It is often assumed that the SAC monitors two distinct events: (1) attachment of kinetochores to microtubules, and (2) tension across the centromere. The latter, would allow the SAC to differentiate between properly bioriented chromosomes, whose centromeres are stretched due to simultaneous attachment of sister kinetochores to the opposite spindle poles, vs. erroneously attached chromosomes (e.g., syntelic - attached to one spindle pole with both kinetochores). However, it has also been suggested that the SAC does not directly monitor tension. Instead, lower stability of erroneous microtubule attachments leads to intermittent reappearance of unattached kinetochores which in turn prevents satisfaction of the SAC. To directly address this issue we examined human cells undergoing mitosis with unreplicated genomes (MUG). Kinetochores in these cells are not paired which implies that the centromere cannot be stretched. However, we find that cells rapidly progress through MUG when kinetochores are attached to microtubules. Nocodazole, taxol, or monastrol arrest MUGed cells indicating that these cells possess an active SAC. Mad2 is recruited to unattached MUG kinetochores and released upon their attachment. In contrast, BubR1 remains on attached kinetochores and exhibits a level of phosphorylation consistent with the inability of MUG spindles to establish normal levels of centromere tension. Thus, attachment of kinetochores to microtubules is sufficient to satisfy the SAC even in the absence of interkinetochore tension. Our data support the view that mitotic exit is not delayed in the presence of erroneous chromosome attachments. Instead, mammalian cells rely on a speedy correction mechanism that makes the delay unnecessary.

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Kinetochore Specification and Function.

A. B. Desai; Ludwig Institute for Cancer Research, La Jolla, CA

We are investigating the molecular mechanisms of kinetochore specification and kinetochore-spindle microtubule attachment. Using in vivo assays in the C. elegans embryo and RNAi-based functional genomics, we identified a set of 5 proteins, including the centromeric histone CENP-A, whose inhibition results in a “kinetochore-null” phenotype. One of these, KNL-2, defined a conserved subfamily of Myb/SANT DNA binding domain-containing proteins specifically required for CENP-A loading. Two of the other KNLs, KNL-1 and KNL-3, are part of a group of 10 interacting kinetochore proteins, referred to as the KMN (KNL-1/Mis12 complex/Ndc80 complex) network. In vivo analysis and in vitro reconstitutions indicate that the KMN network constitutes the core microtubule-binding site of the kinetochore. We propose that multivalent interactions comprised of intrinsically low affinity binding sites within the KMN network generate the dynamic kinetochore-microtubule interface. In collaboration with others, we have visualized the association between the Ndc80 complex and the microtubule lattice using cryo-electron microscopy and demonstrated the ability of this conserved complex to couple movement of objects to depolymerizing microtubules. Additional ongoing projects are focused on centromeric chromatin, kinetochore dynein, correction of errors in chromosome-spindle attachments, spindle checkpoint signaling, and meiosis-specific adaptations in kinetochore assembly.

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Lateral Microtubule Interactions Mediate Chromosome Alignment during Acentrosomal Oocyte Meiosis.

S. Wignall, A. Villeneuve; Developmental Biology, Stanford University, Stanford, CA

In mitotically-dividing cells, duplicated centrosomes are used as structural cues to organize the spindle poles. These centrosomes nucleate microtubules that capture the sister kinetochores of each chromosome, leading to chromosome biorientation and alignment at the metaphase plate. In contrast, in female animals the centrosomes are degraded prior to the meiotic divisions, and therefore oocyte spindles form in their absence. How these acentrosomal spindles are organized and the mechanisms by which chromosomes become bioriented and achieve metaphase alignment are poorly understood. We are using C. elegans oocyte meiosis as an in vivo model for investigating these mechanisms. First, we use high resolution imaging of both bipolar spindles and experimentally-generated monopolar spindles to reveal a surprising organization of microtubules and chromosomes within acentrosomal structures. While bivalents orient axially in bipolar spindles, with the chromosome ends pointing towards the poles, we find that lateral (not end-on) microtubule associations predominate. Bivalents are ensheathed by robust microtubule bundles that run along their sides, whereas microtubule density is very low at chromosome ends despite an abundance of kinetochore proteins encasing these regions of the bivalents. This organization is a special feature of acentrosomal spindles, since centrosome-containing male meiotic spindles appear to have end-on associations. Further, we find that the chromokinesin KLP-19 forms a ring around the center of each bivalent and plays a key role in chromosome congression by providing a polar ejection force. Localization of KLP-19 at the mid-bivalent is coincident with and dependent on the chromosome passenger complex, which is also required for congression. Together, these observations create a new picture of chromosome/microtubule association in acentrosomal spindles and reveal a mechanism by which chromosome alignment can be achieved utilizing this organization, based on chromokinesin-mediated movement along lateral microtubule bundles.

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Polo-like Kinase Regulation of Anaphase Spindle Elongation.

I. Brennan[1], A. Matov[2], G. Danuser[2], A. Straight[1]; [1]Department of Biochemistry, Stanford University School of Medicine, Stanford, CA, [2]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA

The mitotic spindle is a microtubule-based structure responsible for the segregation of chromosomes prior to cellular division. Chromosome separation is achieved through the integration of two processes: anaphase A in which chromosomes are drawn to the spindle pole and anaphase B in which the entire spindle apparatus elongates. We have previously shown that inhibition of polo-like kinase 1 (Plk1) with a small molecule added at anaphase onset prevents anaphase B spindle elongation while not influencing anaphase A. Existing models for anaphase B emphasize the role of microtubule-sliding kinesin motors acting at the spindle midzone to drive the poles further apart. We tested the hypothesis that Plk1 was regulating the activity of a kinesin by depleting an array of spindle-associated motors by RNA-interference. The removal of individual motors or combinations thereof failed to reproduce the Plk1 inhibitor-induced short spindle effect. This is consistent with recent studies of spindle length regulation that have shown little dependence on spindle motors but sensitivity to factors influencing microtubule dynamics. Therefore, we tested whether Plk1 inhibition may block anaphase B by altering microtubule dynamics within the spindle. In order to determine microtubule growth rates, we measured the motion of fluorescent particles of the microtubule tip tracking protein EB1 in live cells. Both kymograph analysis and automated particle tracking show a significant decrease in microtubule growth rate in the presence of Plk1 inhibitor, suggesting a role for polo-like kinase 1 in regulating microtubule dynamics. Furthermore, Plk1 inhibitor-induced spindle shortening is suppressed by stabilizing microtubules through the addition of taxol. Taken together, these data suggest a model for anaphase B in which spindle elongation is robust with respect to removal of microtubule-based motors but sensitive to changes in microtubule dynamics, and polo-like kinase 1 is a key regulator of microtubule growth during anaphase.

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Force and Accuracy for Chromosome Movement in Mitosis.

E. L. Grishchuk[1], I. Spiridonov[1,2], A. K. Efremov[2], V. I. Volkov[1,2], F. I. Ataullakhanov[2,3], J. McIntosh[1]; [1]MCD Biology, University of Colorado, Boulder, CO, [2]National Research Center for Hematology, Moscow, Russian Federation, [3]Center for Theoretical Problems of Physicochemical Pharmacology, Moscow, Russian Federation

Chromosome bi-orientation, the attachment of sister kinetochores to sister spindle poles, is vitally important for accurate chromosome segregation. We have studied this process in vivo by following the congression of pole-proximal kinetochores and their subsequent anaphase segregation in fission yeast cells that carry deletions in any or all of this organism's minus-end directed, microtubule-dependent motors. Although fewer chromosomes segregated normally in these strains, none of these deletions abolished chromosome bi-orientation and segregation, suggesting that microtubule depolymerization is an important part of the mechanism for mitotic motions. Ultrastructural analysis of kinetochore microtubules in this and other cell types suggested that flared protofilaments at microtubule plus ends are coupled to chromosomes by protein complexes that help to transduce depolymerization energy into chromosome motion. We have studied this phenomenon, using an in vitro system to compare the relative effectiveness of brain microtubule-associated proteins, motor enzymes, and kinetochore protein complexes, like Dam1 and Ndc80, for processive cargo motion in association with microtubule shortening. Microtubule depolymerization develops a force against a Dam1 ring that is 6-fold larger than the force exerted on a coupler that binds only one side of a tubulin polymer. Wild type rings slow depolymerization 4-fold, but rings that include a mutant Dam1p with truncated C-terminus slow depolymerization less, consistent with the idea that this tail is part of a strong bond between rings and microtubules, a feature that should facilitate stable attachment and processive kinetochore motion with a shortening microtubule end. Modelling suggests that this "forced walk" mechanism enables the ring to keep a firm grip on disassembling microtubule ends while yielding significant efficiency in energy transduction. We suggest that these features are optimally suited for budding yeast, where each kinetochore is stably attached to only one microtubule.

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The Xenopus TACC3 Protein Maskin Participates in Mitotic Spindle Assembly by Regulating the Association of Microtubules with the Centrosome.

A. Albee, C. Wiese; Department of Biochemistry, University of Wisconsin-Madison, Madison, WI

The centrosome nucleates, anchors, and stabilizes microtubules that are responsible for vesicle and protein transport, cell adhesion, and polarity during interphase, and for mitotic spindle assembly during cell division. Members of the transforming acidic coiled-coil (TACC) family are emerging as important factors in centrosome function and spindle assembly. TACC proteins are found in organisms ranging from yeast to humans and share a conserved ~200 amino acid C-terminal coiled-coil domain. Our studies are focused on the role of the *Xenopus* TACC3 protein, maskin, in centrosome function and mitotic spindle assembly. Depletion of maskin from *Xenopus* egg extracts disrupts microtubule assembly in the extracts and results in the formation of small asters (asters are the initial step in spindle formation in egg extracts). In all species examined thus far, TACC proteins interact with members of the ch-TOG family of microtubule stabilizing proteins. The *Xenopus* ch-TOG protein, XMAP215, stabilizes microtubules by decreasing the catastrophe frequency at microtubule plus ends (Tournebize et al., 2000. Nat. Cell Biol. 2:13). To test the hypothesis that TACCs and TOGs form complexes that stabilize microtubules, we measured the effect of maskin depletion on microtubule stability in egg extracts. Based on the observation that *Drosophila* TACC and TOG proteins oscillate to and from the centrosome (Lee et al., 2001. Nat. Cell Biol. 3:643), we expected maskin depletion to disrupt TOG function and thus decrease microtubule stability, possibly by increasing the catastrophe frequency. Surprisingly, we found that maskin depletion had no effect on microtubule dynamics. Furthermore, we discovered that maskin depletion impairs microtubule anchoring at the centrosome (Albee and Wiese, 2008. Mol. Biol. Cell. 19:3347). Further studies show that maskin, like the *Drosophila* TACC protein, moves with microtubule plus ends. We propose that the association of maskin with microtubule plus ends might serve to regulate maskin protein levels at the centrosome. Our observations lead to a model in which TACC proteins function in spindle assembly by affecting the number of microtubules anchored at the centrosome.

Minisymposium 15: The Nuclear Envelope and Nuclear Pore Complex (792 – 797)

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Lack of Nuclear Pore Complex Turnover in Differentiated Cells Leads to a Loss in Nuclear Compartmentalization.

M. Hetzer; the Molecular and Cell Biology Laboratory (MCBL), the Salk Institute, La Jolla, CA

Nuclear pore complexes (NPCs) are large multi-protein channels that mediate macromolecular transport between the nucleoplasm and the cytoplasm. In dividing cells pores disassemble during mitosis and reassemble into the newly forming nuclei. However, the fate of these macromolecular structures in post-mitotic cells, where the continuous mitotic renewal of pores is absent, is unknown. We show that nuclear pores, unlike other nuclear structures, do not turn over in post-mitotic cells. While a subset of NPC components, like the nucleoporin Nup153, are continuously exchanged at the pore, scaffold nucleoporins such as the Nup107/160 complex, are extremely long-lived and remain incorporated in the nuclear membrane during the entire life span of non-dividing cells. In addition to the lack of nucleoporin expression and NPC turnover, we observed an age-related deterioration of the nuclear permeability barrier and a loss of nucleoporins from old NPCs, suggesting that pore function deteriorates over time in post-mitotic cells.

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Subcellular Positioning of Plant RanGAP1 in Interphase and during Cell Cycle.

I. Meier, J. Brkljacic, X. M. Xu, Q. Zhao, T. Rodrigo-Peiris; Department of Plant Cellular and Molecular Biology, Ohio State University, Columbus, OH

The Ran GTPase plays essential roles in multiple cellular processes, including nucleocytoplasmic transport, spindle formation and post-mitotic nuclear envelope (NE) re-assembly. The Ran GTPase activating protein RanGAP is critical to establish a functional RanGTP/RanGDP gradient across the NE, and is associated with the outer surface of the NE in metazoan and higher plant cells. Association of mammalian RanGAP with the nuclear pore requires a SUMOylated C-terminal domain, which binds to the nucleoporin RanBP2. During mitosis, mammalian RanGAP and RanBP2 have a microtubule-organization function at the kinetochore. In contrast, Arabidopsis RanGAP1 has a unique N-terminal domain, the WPP domain, which is necessary and sufficient for NE targeting. Two distinct, interacting classes of nuclear envelope-associated coiled-coil transmembrane-domain (CC-TMD) proteins are involved in NE-targeting of Arabidopsis RanGAP1 through interaction with the WPP domain. A combination of genetic and biochemical data shows that at least one member of each family is required for RanGAP1 targeting in undifferentiated root cells, while both families are dispensable in other plant tissues, suggesting developmentally controlled RanGAP1 anchoring in plants. During cell cycle in roots, RanGAP1 concentrates at the preprophase band (PPB). The PPB is a plant-specific cortical ring of microtubules and F-actin that predicts the future plane of cell division (cortical division site) and disassembles upon nuclear-envelope breakdown. RanGAP1 remains associated with the cortical division site during mitosis and cytokinesis, requiring the WPP domain but not the two CC-TMD protein families. RanGAP1 persistence at the cortical division site, but not its initial accumulation at the PPB requires the two cytokinesis-regulating kinesins POK1 and POK2, and RanGAP1 and POK1 interact in vivo. Depletion of RanGAP by inducible RNAi leads to phenotypes consistent with cytokinesis defects. The nature of the "molecular memory" that is left behind by the PPB and is proposed to guide the cell plate to the cortical division site is unknown. We propose that RanGAP is a component of this molecular memory and has a role in spatial signalling during plant cell division.

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Artificial Nanopores that Mimic the Transport Selectivity of the Nuclear Pore Complex.

T. Jovanovic-Talisman[1], J. Tetenbaum-Novatt[1], A. S. McKenney[1], A. Zilman[2], R. Peters[3], M. Rout[1], B. T. Chait[1]; [1]The Rockefeller University, New York, NY, [2]Los Alamos National Laboratory, Los Alamos, NM, [3]University of Muenster, Muenster, Germany

Nuclear pore complexes (NPCs) act as effective and robust gateways to the nucleus, allowing only the passage of selected macromolecules across the nuclear envelope. NPCs are comprised of an elaborate scaffold that defines a ~30 nm diameter passageway between the nucleus and cytoplasm. This scaffold anchors proteins termed FG-nups, whose natively disordered domains line the passageway and form an effective barrier to the diffusion of most macromolecules[1]. However, cargo-carrying transport factors overcome this barrier by transient binding to the FG-nups. To test whether nothing more than a passageway and a lining of FG-nups are sufficient for selective transport, we designed a functionalized membrane that incorporates just these two elements. We measured the flux of fluorescently-labelled proteins through the functionalized membranes using a setup inspired by that used for optical single transporter recording of NPCs[2]. Our results demonstrate that this membrane functions as a nanoselective filter, efficiently passing transport factors (NTF2, Kap95, Kap121) and transport factor-cargo complexes (Kap95/IbbGFP, NTF2/RanGDP) that specifically bind FG-nups, whilst significantly inhibiting the passage of proteins that do not bind (ribonuclease A, GFP, BSA, transferrin, IgG). We show that the selectivity is based on the strength of binding to the FG-nup and pore size, as it is *in vivo*[3]. The data also supports our *in silico* prediction that competition between transport factors and nonspecific macromolecules enhances the selectivity of the NPC[4]. This effect has not been a major feature of other models for nuclear transport. In summary, we show that our artificial system recapitulates most key features of trafficking through the NPC, including transport factor-mediated cargo import. Nano-devices of this kind are useful for assessing the significance of parameters that govern NPC gating, and have many potential applications including the purification of macromolecules and pharmaceuticals from crude mixtures. References: 1. Alber, F. et al. Nature **450**, 695, 2007. 2. Kiskin, N. I. et al. Biophys. J. **85**, 2311, 2003. 3. Shulga, N. et al. J. Cell Biol. **149**, 1027, 2000. 4. Zilman, A. et al. PLoS Comput. Biol. **3**, 1281, 2007.

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Nuclear Pore Complex Structure and Assembly Require the ER Membrane Proteins Rtn1 and Yop1.

T. R. Dawson, M. Lazarus, S. Wente; Cell and Developmental Biology, Vanderbilt University, Nashville, TN

Nucleocytoplasmic transport occurs exclusively through nuclear pore complexes (NPCs), macromolecular assemblies embedded in pores formed by inner and outer nuclear membrane fusion events. Although structural studies have resolved the complex NPC architecture, the mechanism that directs pore membrane fusion and NPC biogenesis remains unclear. We identified Rtn1 and Yop1 as potential effectors of pore assembly and/or stability by their mislocalization in the budding yeast prp20-G282S NPC assembly mutant. Reticulons (RTNs) and Yop1/DP1 are conserved membrane protein families required to form and maintain the tubular endoplasmic reticulum (ER). Confocal

microscopy of the prp20-G282S mutant revealed redistribution of Rtn1-GFP and Yop1-GFP to subregions of the cortical ER. Rtn1-GFP and Yop1-GFP also mislocalized to NPC clusters at the nuclear rim in a nup133Δ mutant. We also found that lack of Rtn1 and Yop1 directly perturbed NPC structure. The rtn1Δ yop1Δ mutant cells exhibited Nup mislocalization, NPC clustering, nuclear import defects, and aberrant NPC association with the nuclear envelope. Finally, the rtn1Δ yop1Δ mutant was lethal in combination with mutant alleles for genes encoding Pom34 and Pom152 as well as with members of the Nup84 subcomplex. Remarkably, a rtn1Δ yop1Δ pom34Δ pom152Δ quadruple null mutant was lethal, indicating that Ndc1 was not sufficient alone for nuclear pore assembly. We hypothesize that RTNs and/or Yop1 stabilize the membrane curvature of the nuclear pore during NPC assembly or NPC maintenance.

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A Role for the Nuclear Periphery in the Regulation of Gene Expression.

E. Green, K. Weis; Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Proper organization of the nucleus is required to maintain the appropriate states of gene expression in cells. Chromosomes and protein factors required for gene expression are organized non-randomly within the nucleus to ensure accurate and timely gene expression. Recent studies have demonstrated that some actively transcribed genes localize to the nuclear periphery, contrary to the paradigm that the nuclear periphery is a site of gene silencing and heterochromatin localization. In Saccharomyces cerevisiae, transcriptional activation at multiple genomic loci, including genes that respond to carbon source shifts, heat shock and mating pheromones, correlates with movement of these loci to the nuclear periphery. However, the mechanism of gene movement and its functional significance remains unclear. We analyzed the dynamic movement of the GAL 1-7-10 locus in yeast using a live cell imaging assay, verifying that it does interact with the nuclear periphery more frequently upon transcriptional activation. Interestingly, association of the GAL gene cluster with the nuclear periphery is dependent on actin polymerization, demonstrating a role for actin in long-range chromosome movements. We also investigated the functional consequences of GAL gene localization at the nuclear periphery using mutations that disrupt this interaction. Transcriptional activation, memory of previous transcription states and efficient mRNA export from the nucleus do not appear to depend on an interaction between the GAL gene cluster and the nuclear periphery, contrary to many proposed models. A novel model for the function of the active gene-nuclear periphery interaction will be discussed.

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Asymmetric Segregation of Age during Yeast Budding.

Y. Barral, Z. Shcheprova, S. Baldi, S. Buvelot Frei; Institute of Biochemistry, ETH Zurich, Zurich, Switzerland

A remarkable feature of stem cells resides in their immortality throughout divisions. Similar feature are also observed in unicellular organisms, such as budding yeast, where the mother cells age and finally die, yet generate buds with a full division potential. Thus, the bud lineage, which is immortal, strongly resembles a stem cell. One of the processes that limites the replicative aging of yeast mother cells is the accumulation of extra-chromosomal rDNA circles (ERCs) over generations. ERCs pop out of chromosome XII stochastically due to mitotic recombination between adjacent rDNA repeats. ERCs replicate during S-phase and segregate asymmetrically towards the mother nucleus during anaphase. We investigated the mechanism of ERC retention in yeast mother cells. We show that ERC-related DNA circles localize to the nuclear periphery, where they associate with the basket of nuclear pore complexes (NPCs). Using fluorescence photobleaching techniques, we find that a diffusion barrier forms in the outer membrane of the nuclear envelope as the nucleus passes the bud neck in early anaphase. This barrier prevents most NPCs to pass through the bud neck as the nucleus divides, and thereby confines preexisting pores to the mother cell. Mutants defective in NPC insertion form a daughter nucleus deprived of pores in the bud, while the mother nucleus retains most preexisting pores. Thus, de novo pore insertion is restricted to the bud; mother and bud nuclei are made different relative to the age of their NPCs. Septin and bud-neck mutants that disrupted the diffusion barrier in the nuclear envelope, and pore basket mutant that disrupted the attachment of DNA circles to pores were isolated. In both classes of mutants, DNA circles segregated symmetrically between mother and bud. Furthermore, these mothers aged slower while their daughters were no-longer born young, but instead inherited the age of their mothers. Thus, evolution selected a diffusion-barrier based mechanism to ensure the asymmetric segregation of ageing factors between yeast mother and daughter cells during anaphase. Potential similarities between this process and gametogenesis in higher eukaryotes will be discussed.

Minisymposium 16: Signaling from the Extracellular Matrix (798 – 803)

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Modeling Normal Mammary Gland to Understand Breast Cancer: The Yin and Yang of the ECM and ECM-Degrading Enzymes.

M. Bissell, H. Mori, A. Beliveau, Y. Onodera; Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

The ability of epithelial cells to organize into polarized three dimensional (3D) structures correlates closely with their normal or malignant status. In a versatile model of morphogenesis, we have shown in past studies that inhibiting a number of key signaling pathways in human breast cancer cells grown in laminin-rich ECM gels leads to 'reversion' of the malignant phenotype. The resulting growth-arrested polarized structures resemble normal 'acini' and have helped us to understand how polarity of the normal gland structures may be disrupted as breast cancer progresses. We now have used two additional models to study signaling integration in single mammary cells and also mammary organoids, where we have modeled mammary invasion into the stromal collagen during branching morphogenesis to learn how tumor cells usurp these pathways to invade. We provide additional proof for our contention that all signaling pathways must directly or indirectly communicate to maintain homeostasis. We show how biochemical and mechanical signaling from the ECM, the ECM receptors and MMPs interconnect with cell and tissue architecture in reciprocal and reiterating loops in tissue specificity. Acknowledgments: The work from MJB's laboratory is supported by grants from the OBER Office of Biological and Environmental Research of the U.S. Department of Energy (DE-AC02-05CH11231), a Distinguished Fellow Award; Low Dose Radiation Program and the Office of Health and Environmental Research, Health Effects Division, U.S. Department of Energy (03-76SF00098); by National Cancer Institute awards R01CA064786, R01CA057621 and U54CA126552.

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CD47 and Thombospondin-1 Limit Mitochondrial Biogenesis.

E. Frazier[1], J. Isenberg[3], L. Pappan[1], M. Abu-Asab[3], M. Tsokos[3], D. Roberts[3], K. Green[2], R. Schmidt[2], W. A. Frazier[1]; [1]Biochemistry, Washington University, St. Louis, MO, [2]Pathology, Washington University, St. Louis, MO, [3]Laboratory of Pathology, NCI-NIH, Bethesda, MD

Thrombospondin-1 (TSP1), a "matricellular" protein, and its receptor, CD47, constitute a newly discovered regulatory system that limits endogenous nitric oxide (NO) signaling. Mice lacking either TSP1 or CD47 are protected from tissue ischemia and reperfusion injury, resist endothelial dysfunction with aging and have elevated levels of cGMP in several tissues. All these effects are indicative of enhanced NO signaling. A recently appreciated function of NO/cGMP is regulation of mitochondrial biogenesis through control of the level of the transcriptional coactivator PGC-1α, which acts with other factors to coordinate mitochondrial biogenesis and energy metabolism. Ultrastructural studies reveal that tissues from young CD47- and TSP1-null mice (2-3 months old) have dramatically elevated numbers of mitochondria in skeletal muscle, but a near normal content of mitochondria in heart tissue. Running endurance of the CD47-null mice is superior to WTs. Quantitative RT-PCR and Western blotting of mitochondrial markers confirm the tissue-specific shift in mitochondrial content in the CD47-nulls vs WTs. Cultured primary cells from CD47-null animals have more mitochondria than WT counterparts, and cell lines lacking CD47 have fewer mitochondria when CD47 is expressed via transfection. Whole animal respirometry (Oxymax) reveals that CD47-nulls use less oxygen and have a higher respiratory exchange ratio than matched WTs, indicative of a preferential use of fatty acids (via mitochondrial β-oxidation) vs carbohydrates as their primary energy source. Consistent with enhanced fatty acid oxidation, the CD47-nulls remain leaner than WTs when fed either a low fat chow diet or a high fat "Western diet". Their lower body weight appears to be accounted for by decreased visceral fat. As CD47-null mice age, their skeletal muscle mitochondrial content declines to near WT levels. In contrast, the mitochondrial content of CD47-null cardiac myofibrils dramatically increases compared to WTs. In summary, CD47 signaling limits mitochondrial biogenesis, favors obesity and may exacerbate the effects of aging on the cardiovascular system.

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Hyaluronan-CD44 Interaction Activates Stem Cell-specific Nanog Signaling, Stat-3-Mediated MDR1 Gene Expression and Ankyrin-regulated Multidrug Efflux in Breast and Ovarian Tumor Cells.

L. Bourguignon, K. Peyrollier, W. Xia, E. Gilad; Department of Medicine and VA Medical Center, University of California at San Francisco, San Francisco, CA

Hyaluronan (HA) is a major glycosaminoglycan in the extracellular matrix whose expression is tightly linked to multidrug resistance and tumor progression. In this study we investigated HA-induced interaction between CD44 (a HA receptor) and Nanog (an embryonic stem cell transcription factor) in both human breast tumor cells (MCF-7 cells) and human ovarian tumor cells (SK-OV-3.ipl cells). Using a specific primer pair to amplify Nanog by RT-PCR, we detected the expression of Nanog transcript in both tumor cell lines. In addition, our results reveal that HA binding to these tumor cells promotes Nanog protein association with CD44 followed by Nanog activation, nuclear translocation and the expression of pluripotent stem cell regulators (e.g., Rex1 and Sox2). Nanog also forms a complex with the "signal transducer and activator of transcription protein 3" (Stat-3) in the nucleus leading to Stat-3-specific transcriptional activation and multidrug transporter, MDR1 (P-glycoprotein/P-gp) gene expression. Furthermore, we observed that HA-CD44 interaction induces ankyrin (a cytoskeletal protein) binding to MDR1 resulting in the efflux of chemotherapeutic drugs [e.g., Doxorubicin and Paclitaxel (taxol)] in these tumor cells. All of these events then contribute to tumor cell growth and multidrug resistance in both breast and ovarian tumor cells. Overexpression of Nanog by transfecting tumor cells with Nanog cDNA stimulates Rex1/Sox2 gene expression and Stat-3-transcriptional activation as well as tumor cell behaviors (e.g., MDR1 overexpression, and multidrug resistance). Downregulation of Nanog signaling or ankyrin function [by transfecting tumor cells with Nanog siRNA or ankyrin repeat domain (ARD)cDNA] not only blocks HA/CD44-mediated tumor cell behaviors but also enhances chemosensitivity. Taken together, these findings suggest that targeting HA/CD44-mediated Nanog-Stat-3 signaling pathways and ankyrin/cytoskeleton function may represent a novel approach to overcome chemotherapy resistance in some breast and ovarian tumor cells displaying stem cell-like properties during tumor progression.

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Induction of Cell Polarization and Migration by a Gradient of Nanoscale Variations in Adhesive Ligand Spacing.

J. P. Spatz[1], V. Hirschfeld-Warneken[1], B. Geiger[2]; [1]New Materials and Biosystems, Max Planck Institute for Metals Research & University of Heidelberg, Stuttgart, Germany, [2]Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel

Cell interactions with adhesive surfaces play a vital role in the regulation of cell proliferation, viability and differentiation, and affect multiple biological processes. Since cell adhesion depends mainly on the nature and density of the adhesive ligand molecules, spatial molecular patterning, which enables the modulation of adhesion receptor clustering, might affect both the structural and signalling activities of the adhesive interaction. We herein show that cells plated on surfaces that present a molecularly defined spacing gradient of an integrin RGD ligand, can sense small but consistent differences in adhesive ligand spacing of about 1 nm across the cell diameter, which is approximately 61 µm when the spacing includes 70 nm. Consequently, these positional cues induce cell polarization, and initiate cell migration and signalling. We propose that differential positional clustering of the integrin transmembrane receptors is used by cells for exploring and interpreting their environment, at high spatial sensitivity.

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Control of ECM Signaling by a Tension Activated Fibronectin Switch.

D. Boettiger[1], M. Lee[1], J. Friedland[1], M. Chan[1], T. Miller[2]; [1]Microbiology, University of Pennsylvania, Philadelphia, PA, [2]Exercise Science, George Washington University, Washington, DC

Signaling from the extracellular matrix is defined by its interaction with cell surface adhesion receptors (integrins) yet the relationship between this step and the intracellular signals remains the least understood element in the process. The extracellular matrix itself is commonly assigned a passive role in the signaling process. Here we show that stimulation of FAK Y397 phosphorylation as a consequence

of α5β1 integrin binding to fibronectin requires a tension-dependent change in fibronectin conformation. Constraining fibronectin conformation by adsorption to uncharged surfaces does not affect either the number of strength of α5β1-fibronectin adhesive bonds that form, but this adhesion fails to elicit phosphorylation of FAK Y397. Adding a negative charge to the surface prevents the negatively charged cell binding domain from interacting with the surface thus releasing the constraint to the fibronectin conformational switch and allowing FAK phosphorylation. The dependence of this process on tension was demonstrated in two ways. First, blocking the ability of myosin II to tension actin blocked FAK phosphorylation. Second, attaching the fibronectin to a soft substrate that has less resistance to the force applied by myosin also blocked phosphorylation of FAK. Neither of these treatments affected the number of α5β1-fibronectin adhesive bonds. Genetic analysis showed that this signaling was dependent on R1374 and R1379 in the synergy domain of fibronectin. These data demonstrate that fibronectin acts as conformational switch within a complex that contains myosin II, actin, talin, and α5β1 integrin to control integrin-mediated signaling. This complex would mediate cellular responses to differences in substrate stiffness and provide a mechanism for tensional homeostasis.

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Talin Regulation of Integrin Clustering and Signaling.

B. Wehrle-Haller[1], F. Saltel[1], P. Pinon[1], M. Jacquier[1], W. Liu[2]; [1]Cell Physiology & Metabolism, University of Geneva, Centre Medical Universitaire, Geneva, Switzerland, [2]Biosciences and Nutrition, Novum, Karolinska Institutet, Huddinge, Sweden

Integrins provide not only physical interaction with the extracellular matrix, but create also cellular signaling when bound to their specific extracellular ligands. Despite the importance of the signaling process, its mechanism is not understood. Integrin function requires a metal-ion dependent adhesion site in the extracellular domain and cytoskeletal adaptor binding sites in the cytoplasmic domain of the beta-subunit. One of these sites, the membrane proximal NPXY motif, has been established as a binding site for the head-domain of the cytoplasmic adaptor talin, mediating integrin activation, a prerequisite for integrin ligand-binding. Furthermore, the talin-head domain induces integrin clustering in cells exposed to ligand-coated surfaces. Employing fluorescent-tagged integrins and site directed mutagenesis, we have identified membrane-proximal acidic amino acids required for talin-head dependent integrin clustering. By using a charge-inversion complementation assay, we determined basic residues, located within a loop in the talin F3 subdomain as the integrin binding site. Surprisingly, the talin-head/integrin clustering occurred in the absence of a functional NPXY motif, as long as integrin activation was achieved by mutagenesis or manganese treatment. Based on docking studies of the integrin peptide to the talin surface and the differential requirement of the NPXY motif for integrin activation and clustering, we propose that the talin/integrin interaction matures from a recruitment/activation (NPXY-dependent) to a clustering/signaling (NPXY-independent) state. The shift in integrin/talin association results in NPXY exposure, liberating this motif for signaling. In accordance with this hypothesis, the expression of a NPXY mutant integrin caused a dominant negative effect on integrin- and integrin/growth factor-dependent cell spreading. This inhibition of spreading required a functional extracellular-ligand binding site, proposing the formation of ligand- and talin-bound integrin complexes with exposed NPXY motifs that are required for signaling during cell spreading and focal adhesion formation.

Peroxisomes (804 – 811)

804/B1

Peroxisome Deficiency Causes a Complex Phenotype Due To Hepatic SREBP/Insig Dysregulation Associated with Endoplasmic Reticulum Stress.

W. J. Kovacs[1,2], K. N. Tape[2], J. E. Shackelford[2], T. M. Wikander[5], M. J. Richards[3], S. Fliesler[3,4], S. K. Krisans[2], P. L. Faust[5]; [1]Cell Biology, Swiss Federal Institute of Technology Zuerich (ETH Zuerich), Zuerich, Switzerland, [2]Biology, San Diego State University, San Diego, CA, [3]Saint Louis University Eye Institute and Department of Pharmacological and Physiological Science, Saint Louis University School of Medicine, St. Louis, MO, [4]Ross Eye Institute / Vision Research Center, VA Western New York Healthcare System, SUNY - University at Buffalo, Buffalo, NY, [5]Pathology, Columbia University, New York, NY

Regulation of hepatic cholesterol biosynthesis, lipogenesis and insulin signaling intersect at the transcriptional level by control of *SREBPs* and *Insig* genes. We previously demonstrated that peroxisome-deficient *PEX2-/-* mice activate *SREBP-2* pathways, but are unable to maintain normal cholesterol homeostasis. In this study, we demonstrate that oral bile acid treatment normalized hepatic and plasma cholesterol levels and hepatic cholesterol synthesis in early postnatal *PEX2* mutants, but *SREBP-2* and its target gene expressions remained increased. *SREBP-2* pathway induction was also observed in neonatal and longer surviving *PEX2* mutants, where hepatic cholesterol levels were normal. Abnormal expression patterns for *SREBP-1c* and *Insig-2a*, and novel regulation of *Insig-2b*, further demonstrate that peroxisome deficiency widely affects the regulation of related metabolic pathways. We show that peroxisome deficiency activates hepatic endoplasmic reticulum (ER) stress pathways, especially the integrated stress response, suggesting a mechanism whereby ER stress leads to dysregulation of the endogenous sterol response mechanism. The exact mechanisms triggering ER stress in peroxisome-deficient *PEX2-/-* livers are unclear but likely involve multiple signals, including perturbed flux of mevalonate metabolites, changes in fatty acid levels and composition, and oxidative stress. Our results highlight the importance of functional peroxisomes for maintaining metabolic homeostasis and the overall pathology in the *PEX2-/-* mouse model has potential implications for future disease treatment interventions that target lipid metabolism.

805/B2

Characterization of Cellular Signaling Networks Governing Responses to Fatty Acid Exposure.

R. Saleem[1], B. Knoblach[2], R. Rogers[1], F. Mast[2], A. Jamakhandi[1], J. Boyle[1], J. Smith[1], R. Rachubinski[2], J. Aitchison[1,2]; [1]Institute for Systems Biology, Seattle, WA, [2]Cell Biology, University of Alberta, Edmonton, AB, Canada

Objective: To use quantitative imaging, network analysis and quantitative phosphoproteomics to elucidate the dynamic signaling networks that govern the response of Saccharomyces cerevisiae to fatty acid exposure and the concomitant biogenesis of peroxisomes. Results: We have used systems approaches to uncover signaling networks that regulate the response of S. cerevisiae to fatty acid exposure. Specific

networks of signaling molecules function to regulate repression, derepression, and induction of fatty acid responsive loci, as well as peroxisome morphology. This study represents the first comprehensive investigation of kinase and phosphatase activity in the complex biological process of fatty acid metabolism and organelle biogenesis. We have begun to quantitatively assay the temporal phosphorylation status of the proteome in response to fatty acid stimulation to delineate information flow within these signaling networks. We have used IMAC enrichment of phosphopeptides to identify over three hundred phosphoproteins in various conditions and are now using HILIC based fractionation to increase our ability to detect phosphopeptides. An analysis of the phosphoproteome at several different time points will provide maps of the early, mid and late signal transduction events that occur during the response and biogenesis program of peroxisomes. Conclusions: We have mapped the signaling molecules and networks regulating repression, derepression and induction of fatty acid responsive loci. We have also established protocols and performed a comprehensive characterization of the S. cerevisiae phosphoproteome under various conditions of repression and activation of the response. These experiments will provide a temporal, detailed view of these signaling networks and an extensive understanding of the signaling that regulates fatty acid metabolism and peroxisomes biogenesis. These studies will not only illuminate the biology underlying the cellular response to fatty acids, potentially informing human fatty acid/peroxisomal disorders, but will also establish methods that can be expanded to map the signaling pathways in response to a host of stimuli in both yeast and mammalian cells.

806/B3

A Novel Import and Trafficking Pathway for the Peroxisomal Membrane Protein, Pex30p, in Saccharomyces cerevisiae.

F. D. Mast, R. J. Perry, R. Rachubinski; Cell Biology, University of Alberta, Edmonton, AB, Canada

Understanding how peroxisomes originate at the ER, the starting point for eukaryotic cell secretion, is key to elucidate why loss of peroxisome function results in the lethal congenital disorders collectively known as the Peroxisome Biogenesis Disorders. An important goal, therefore, is to identify how peroxisomal membrane proteins are inserted into the ER and subsequently delivered to peroxisomes. We define such a pathway for Pex30p, a recently identified peroxisomal membrane protein that contains a dysferlin motif and is involved in the regulation of peroxisome growth and division in the budding yeast, *Saccharomyces cerevisiae*. Confocal fluorescence colocalization microscopy showed that Pex30p localizes to subdomains of the ER when yeast are grown under peroxisome-repressing conditions but moves to peroxisomes upon peroxisome-induction with oleate. The ER localization of Pex30p was confirmed using a biochemical "ER-shift" assay in which Pex30p resolved to the same fractions as the Sec61p translocon. Furthermore, Pex30p is targeted to the ER in the absence of Pex3p and does not require the assistance of the cytosolic peroxin-chaperone protein, Pex19p, to be inserted into the ER. Using truncated fluorescent chimeras of Pex30p, we find the N-terminal 282 amino acids of Pex30p are sufficient for its targeting to, and insertion into, the ER. Finally, Pex30p-GFP mislocalizes to and accumulates in the vacuole at the nonpermissive temperature of a *sec61*^{ts} strain expressing a mutant form of the principal ER translocon protein, Sec61p. Our results support a scenario in which at least some peroxisomal membrane proteins are capable of maintaining an ER distribution when peroxisomes are not needed. This steady-state localization to the ER of a peroxin involved in regulating the size and number of peroxisomes reveals an unexpected role for the ER in regulating peroxisome homeostasis. This is the first study to identify a role for the Sec61p translocon in peroxisome biogenesis by inserting a peroxisomal membrane protein into the ER.

807/B4

Peroxisome Membrane Biogenesis: The Peroxisomal Membrane Transport Receptor Pex3p is Directly Imported Via Pex16p in a Pex19p-dependent Manner.

Y. Fujiki[1,2], T. Matsuzaki[1]; [1]Department of Biology, Faculty of Sciences, Kyushu University Graduate School, Fukuoka, Japan, [2]JST, Crest, Tokyo, Japan

Peroxisomal matrix and membrane proteins (PMPs) are synthesized on free polysomes in the cytosol and post-translationally imported into peroxisomes (Lazarow & Fujiki, 1985). Genetic phenotype-complementation assays of peroxisome-membrane-deficient mutants of yeast and mammalian cells led to the isolation of Pex3p, Pex16p, and Pex19p, which are all indispensable for PMP import. In contrast to the matrix protein import, the molecular mechanisms underlying the assembly of PMPs remain poorly defined. Two distinct import pathways of PMPs have recently been proposed. Pex19p forms complexes with newly synthesized class-I PMPs excluding Pex3p in the cytosol, playing roles as a chaperone and a transporter. Pex3p functions as the membrane receptor for the Pex19p-PMP complexes, thereby PMPs are imported. However, the molecular mechanism of the biogenesis of class-II Pex3p remains elusive. We herein show that Pex19p also forms a soluble complex with newly synthesized Pex3p in the cytosol and translocates it to peroxisomes. Knocking down of Pex19p results in a failure of peroxisomal localization of Pex3p. Furthermore, we demonstrate that Pex16p serves as the membrane receptor specific to the Pex3p-Pex19p complexes on the peroxisomal membranes. Based on these novel findings, we suggest a model for import of PMPs, termed class-II PMP import dependent on Pex16p and Pex19p, providing new insights into the molecular mechanisms underlying the biogenesis of peroxisomes and its regulation involving Pex3p, Pex19p, and Pex16p. (Supported by CREST, JST grant, Grant-in-Aid for Scientific Research and 21COE & Global COE Programs, Japan)

808/B5

Etherphospholipids, Plasmalogens Are Transported via an ATP-dependent and Non-vesicular Pathway: Study with Plasmalogen-deficient CHO Cell Mutant.

Y. Yagita[1], M. Honsho[2], N. Kinoshita[2], Y. Fujiki[1,2,3]; [1]Graduate School of Systems Life Sciences, Kyushu University, Fukuoka, Japan, [2]Department of Biology, Faculty of Sciences, Kyushu University, Fukuoka, Japan, [3]JST, CREST, Tokyo, Japan

Plasmalogens are a major sub-class of ethanolamine and choline phospholipids in which sn-1 position has a long chain fatty alcohol attached through a vinyl ether bond. The first two steps of plasmalogen biosynthesis are catalyzed by peroxisomal enzymes and the following steps take place in the endoplasmic reticulum. Physiological importance of plasmalogens is inferred from the findings that plasmalogens are severely deficient in specimens from patients with various genetic degenerative disorders such as Zellweger syndrome and rhizomeric chondrodysplasia punctata. However, functional roles and subcellular localization of plasmalogens remain elusive. We herein isolated a

plasmalogen-deficient Chinese hamster ovary cell mutant, ZPEG251, with a phenotype of normal import of peroxisomal matrix and membrane proteins. In ZPEG251, ethanolamine plasmalogen (PlsEtn) was severely reduced. Complementation analysis by expression of genes responsible for the plasmalogen biosynthesis suggested that alkyl-dihydroxyacetonephosphate synthase (ADAPS), catalyzing the second step of plasmalogen biogenesis, was deficient in ZPEG251. ADAPS mRNA was barely detectable in total RNA from ZPEG251 as verified by Northern blot and reverse transcription-PCR analyses. Defect of ADAPS expression was also assessed by immunoblot. As a step toward delineating functional roles of PlsEtn, we investigated its subcellular localization and transport pathway. PlsEtn was localized to post-Golgi compartments and enriched in detergent-resistant membranes. Transport of PlsEtn to post-Golgi compartments was affected by lowering cellular ATP, but not by inhibitors of microtubule assembly and vesicular transport, implying the existence of an ATP-dependent and non-vesicular pathway of PlsEtn transport where an unidentified lipid transporter(s) might be involved.

809/B6

Peroxisome Fission in Hansenula Polymorpha Requires Mdv1 and Fis1 Two Proteins also Involved in Mitochondrial Fission.

S. Nagotu[1], A. M. Krikken[1], M. Otzen[1], J. Kiel[1], M. Veenhuis[1,2], I. Van der Klei[1,2]; [1]Molecular Cell Biology, Groningen Biosciences and Biotechnology Institute, University of Groningen, Haren, Netherlands, [2]Kluyver Centre for Genomics of Industrial Fermentation, Kluyver Centre, Delft, Netherlands

Peroxisomes are single membrane bound highly dynamic organelles present in eukaryotic cells. They display a remarkable versatility with respect to their function depending on organism and growth conditions. Recent years have witnessed several new findings with respect to peroxisome biology making the research on peroxisomes strongly interesting and challenging. One such challenging topic of the field concerns the biogenesis of the organelles. Studies from several groups have now convincingly shown that peroxisomes may behave like autonomous organelles or can be formed from endoplasmic reticulum (Hoepfner et al, 2005). Our work aimed at elucidating the proteins required for division of peroxisomes in the methylotrophic yeast H.polymorpha. Membrane fusion and fission events in a yeast cell are governed by three dynamin-like proteins Vps1, Dnm1 and Mgm1. We have observed that H.polymorpha predominantly has a Dnm1 dependant peroxisome fission machinery unlike Saccharomyces cerevisiae which requires both Vps1 and Dnm1 (Kuravi et al, 2006; Motley et al. 2007). Peroxisomes in cells lacking Dnm1 form long extensions into the daughter cell that most likely divide during cytokinesis (Nagotu et al, 2008). Various data indicated that Dnm1 is required for mitochondrial division and is present in a complex with Fis1, Mdv1/Caf4. Quantitative analysis of H. polymorpha Fis1 and Mdv1 deletion mutants revealed a strong reduction in peroxisome numbers at inducing growth conditions. Caf4, the paralog of Mdv1, was not identified in H. polymorpha. Dnm1-mCherry and GFP-Mdv1 were observed to co-localize on peroxisomes apart from mitochondria in WT cells. However, Dnm1-GFP distribution pattern was affected in Mdv1 and Fis1 deletion mutants. The number of Dnm1 puncta were drastically reduced in these cells but were still observed to be associated to organelles. Organelle associated Dnm1 in these cells could be non-functional as overexpression of Dnm1 in these cells does not lead to enhanced peroxisome proliferation as observed in WT cells. Taken together these data indicate that in H. polymorpha peroxisome and mitochondrial fission is mediated by a similar organelle fission machinery.

810/B7

YlPex3Bp is a Peroxisomal Membrane Protein Involved in Peroxisome Inheritance and Morphology in Yarrowia lipolytica.

J. Chang, F. Mast, G. Eitzen, R. Rachubinski; Cell Biology, University of Alberta, Edmonton, AB, Canada

In *Saccharomyces cerevisiae*, the class V myosin motor, Myo2p, interacts specifically with its peroxisomal receptor, Inp2p, to move peroxisomes along actin from mother cell to bud. We previously showed that peroxisome mobility and inheritance in the dimorphic yeast *Y. lipolytica* are also dependent on the actin cytoskeleton. Interrogation of the *Y. lipolytica* genome revealed the presence of only one class V myosin. This myosin V is involved in transporting peroxisomes from mother cell to bud in *Y. lipolytica*. We identified *Yl*Pex3Bp, a paralog of *Yl*Pex3p and a peroxisomal integral membrane protein, as the peroxisome-specific receptor for myosin V in *Y. lipolytica*. *Yl*Pex3Bp shows no homology to Inp2p. *Yl*Pex3Bp interacts directly with the globular tail of myosin V. *Yl*Pex3Bp also interacts with itself and with *Yl*Pex3p. In cells lacking *Yl*Pex3Bp, peroxisomes were preferentially retained in the mother cell, while, the majority of peroxisomes gathered and were transferred to the bud in cells overexpressing *Yl*Pex3Bp. Overexpression of *Yl*Pex3p can partially complement the phenotype of *Ylpex3B Δ* , while overexpression of *Yl*Pex3Bp cannot complement the phenotype of *Ylpex3 Δ* . Interestingly, cells lacking *Yl*Pex3Bp contain hyperelongated, tubulo-reticular peroxisomes. Therefore, *Yl*Pex3Bp also has a role in peroxisome morphology. Our data suggest that *Yl*Pex3Bp is a multifunctional protein that is involved in the control of both peroxisome inheritance and morphology in *Y. lipolytica*.

811/B8

An Organellar Tether Required for Woronin Body Biogenesis.

S. K. Ng, F. Liu, J. Lai, G. Jedd; Temasek Life Sciences Laboratory, Singapore, Singapore

Woronin bodies (WBs) are dense-core peroxisomal organelles found exclusively in the filamentous Ascomycetes (Pezizomycotina), where they perform an adaptive function to seal the septal pore in response to cellular wounding. WBs are centered on a self-assembled matrix protein, HEX, and differentiate from peroxisomes through the WSC membrane protein. WSC envelops HEX assemblies to promote their budding and is also required for WB inheritance by cell cortex association, however, the link between WSC and cortex is poorly understood. Here, we describe the isolation and preliminary characterization of Leashin, a new component of the WB segregation machinery. Leashin mutants accumulate nascent WBs in the apical hyphal compartment. N-terminal sequences of Leashin bind WBs via WSC and discreet C-terminal sequences are required for association with the cell cortex. Together, our data suggest that Leashin functions as an organellar tether. Additional evidence derived from comparison of WB tethering in different fungal species suggests that variation in Leashin may determine evolutionary variation in patterns of cortex association.

Endosomes and Lysosomes (812 – 824)

812/B9

Lysosomal Acid Lipase Inhibitors Identified from Cell-based Screens Reduce Sterol Accumulation in Niemann-Pick Type C Cells.

A. I. Rosenbaum[1], M. Rujoi[2,3], A. Y. Huang[2], N. Pipalia[2], F. Maxfield[2]; [1]Program in Biochemistry and Structural Biology, Weill Graduate School of Medical Sciences of Cornell University, New York, NY, [2]Department of Biochemistry, Weill Medical College, New York, NY, [3]Current address: Howard Hughes Medical Institute, University of Chicago, Chicago, IL

Niemann-Pick disease type C (NPC) is a fatal autosomal recessive lysosomal storage disorder causing abnormal accumulation of unesterified cholesterol and other lipids in cells. This phenomenon can be visualized by filipin staining of the lysosomal storage organelle (LSO). Human NPC disease is caused by mutations in NPC1, a large transmembrane, late endosomal /lysosomal protein or in NPC2, a soluble, cholesterol-binding protein that is targeted to the lumen of late endosomes by a mannose-6-phosphate modification. No effective therapy currently exists for either type of NPC. High throughput automated microscopy screens in both human and hamster NPC1 cells [1] have identified a series of small molecule compounds that partially diminish the NPC phenotype in cell culture. We are identifying the molecular targets and mechanism of action of these compounds. We have found that about 1/3 of the most effective compounds identified in a screen of a CHO cell line with an NPC1 defect were inhibitors of lysosomal acid lipase (LAL), the enzyme that hydrolyzes LDL-derived triglycerides and cholesteryl esters. These compounds inhibited both cholesteryl ester hydrolysis in cells and LAL activity in enzymatic assays using cell lysates. Interestingly, there was remarkable species specificity in this inhibition. None of the compounds that were potent inhibitors of CHO-derived LAL were effective inhibitors of human LAL activity. However, two of the most potent hits from a screen of human NPC1 cells are potent inhibitors of both CHO and human LAL. Both of these compounds effectively reduce cholesterol accumulation in LSOs of both cell types. RNAi knockdown of LAL in human NPC1 fibroblasts recapitulated these results, confirming that LAL was the target of these compounds. This work demonstrates use of small molecule phenotypic cellular screens to identify mechanistically plausible cellular targets and further elucidate such a complicated cellular process as cholesterol homeostasis in cultured cells. This work may provide direction for therapeutics development to treat NPC disease and other diseases associated with cholesterol transport. 1. Pipalia, N.H., et al., J Lipid Res, 2005.

813/B10

Nanogold-IgY-Nanophosphor Tagged Approach for Affinity Retrieval of Lysosomal Membrane Transporter Proteins -A Proteomics Based Approach to Study on Lysosomal Storage Diseases.

D. K. Gupta[1], S. Sundaram[2], S. Srivastava[2], E. Smolenova[3], A. Hasilik[3]; [1]Biochemistry & Center of Bioinformatics, IIDS, University of Allahabad, Allahabad, India, [2]Center of Biotechnology, IIDS, University of Allahabad, Allahabad, India, [3]Institute of Physiological Chemistry, University of Marburg, Marburg, Germany

We are employing a proteomics based approach to study molecular basis of lysosomal storage diseases resulting from defects of membrane transporter proteins. The proteo-mics data on amino acid sequence targeting motifs in cytosolic domains in lysosomal membrane proteins are employed that allow for a design of antibodies (IgYs) against these to be raised in hens and collected from egg yolk. These IgYs adsorbed onto 40 nm nanogold particles to make nonogold -IgY conjugates and subsequently coated with nonophosphors can be employed to retrieve lysosomal tranport vesicles from Human lymphocytic U937 cells by a combination of affinity or density perturbation and subcellular density-gradient ultracentrifugation protocol. Purification of Control Antibodies (IgY) of hen origin has been standardized in our lab. cDNAs Reconstructs made from available cDNAs against various membrane transporters (Prof.Andrej Hasilik, Universitaet Marburg, Dr.S.Wiezmann, DKFZ) were cloned in 2 vectors, pCI and pcDNA3 and used in transfection experiments involving cultured human HEK 293 cells separately with CFP(Cyan variant mutant of Green Fluorescent Protein) and YFP(Yellow variant mutant of GFP).We studied immunofluorescence using anti-Cathepsin D Ig (as control lysosomal visualization) and anti-GFP Ig to localize the CFP- and YFP-fusion protein (DKFZ-p564K2464). The transfection seems to be at a lower rate, upto 30%. Atleast 50 % transfection efficiency to improve the results is needed. We have evaluated the expression of the fusion protein GFP- DKFZ-p564K2464, in 2 metabolic labeling experiments with the transfected HK 293 cells from the same batches in parallel. The recombinant protein was immunoprecipitated from cell lysates indicating that the cDNA for hypotheti-cal membrane protein is indeed made and processed by the cells and is a real one. Retrieval of lysosomal membrane transporter proteins using a double tag , i.e. Nanogold-IgY -Nanphosphor Conjugate - can provide an insight into the molecular basis of lysosomal storage diseases.

814/B11

Characterization and Comprehensive Proteome Profiling of Exosomes Secreted by Hepatocytes.

J. Conde-Vancells[1], E. Rodriguez-Suarez[1], N. Embade[1], D. Gil[1], E. Gonzalez[1], R. Matthiesen[1], F. Elortza[1], S. C. Lu[2], J. M. Mato[1], J. M. Falcon[1]; [1]CIC bioGUNE, CIBERehd, Derio, Spain, [2]Division of Gastrointestinal and Liver Diseases, University Southern California, Los Angeles, CA

Exosomes represent a discrete population of vesicles that are secreted from various cell types to the extracellular media. Their protein and lipid composition are a consequence of sorting events at the level of the multivesicular body, a central organelle which integrates endocytic and secretory pathways. Characterization of exosomes from different biological samples has shown the presence of common as well as cell-type specific proteins. Remarkably, the protein content of the exosomes is modified upon pathological or stress conditions. Hepatocytes play a central role in the body response to stress metabolizing potentially harmful endogenous substances as well as xenobiotics. In the present study we described and characterized for first time exosome secretion in non-tumoral hepatocytes, and using a systematic proteomic approach, we establish the first extensive proteome of a hepatocyte-derived exosome population which should be useful in furthering our understanding of the hepatic function and in the identification of components that may serve as biomarkers for hepatic alterations. Our analysis identifies a significant number of proteins previously described among exosomes derived from others cell types as well as proteins involved in metabolizing lipoproteins, endogenous compounds and xenobiotics, not previously described in exosomes. We also demonstrated that hepatocyte-derived exosomes express the hepatic-specific receptors which can be used to isolate these specific exosomes in complex biological samples (e.g. blood) which contain exosome populations secreted by different cell-types. Furthermore, we demonstrated that exosomal membrane proteins can constitute an interesting tool to express non-exosomal proteins into exosomes with therapeutic purposes.

815/B12

Functional and Biochemical Characterization of Rab7 Mutants Responsible of Charcot-Marie-Tooth Type 2B.

A. De Luca, C. Progida, M. R. Spinosa, A. M. Colucci, P. Alifano, C. Bucci; Di.S.Te.B.A., Università del Salento, Lecce, Italy

Hereditary motor and sensory neuropathies constitute a group of pathologies of the peripheral nervous system. Charcot-Marie-Tooth (CMT) is one of the most common disease, affecting 1:2500 individuals. The type 2B (CMT2B) has an autosomal dominant inheritance and is clinically characterized by marked distal muscle weakness, atrophy, sensorial loss and limbs deformity with a high frequency of foot ulcers and recurrent infections leading to amputations. For this reason these diseases are also called "ulcero-mutilating neuropathies". Four mutations in the gene encoding Rab7 protein have been identified as causative for CMT2B. These mutations affect highly conserved aminoacidic residues. Rab7 protein is a member of the RAB family of RAS-related GTP-binding proteins and is ubiquitous. Thus, how these mutants damage only peripheral motor and sensory nerves is unclear. The aim of this work is to characterize these mutants in order to understand the molecular mechanism responsible for their phenotypes. As Rab7 is a GTPase, we characterized biochemically the mutants: we found that they are able to bind GTP although they can idrolize it much more slowly than the wt. We also compared the nucleotide dissociation activity of the mutants to that of the wt form and we showed that Koff values of the mutants were higher than that of Rab7 wt, proving that the mutants have reduced affinity for guanine nucleotides, in particular for GDP. Furthermore, we evaluated the GTP:GDP ratio in the mutant proteins expressed in HeLa cells: the mutants were found mainly in the GTP bound form (87%-92%) unlike wt Rab7 (23%). To investigate if Rab7 function is modified in presence of the mutations, since Rab7 activity influences EGF and EGFR degradation, we performed EGF and EGFR degradation assay in HeLa cells. Expression of the CMT2B Rab7 mutant proteins did not alter EGF or EGFR degradation. Furthermore, we tested the ability of the mutants to rescue Rab7 function in Rab7-depleted cells. All the CMT2B mutants were able to restore EGFR degradation indicating that they are active mutants. In conclusion, we showed that these mutants have modified biochemical properties and are "gain of function" mutants.

816/B13

Sorting Nexin 27: a Novel Regulator of CFTR Trafficking.

M. I. McDermott[1], W. R. Thelin[3], P. Lyons[1], M. Gentzsch[3], W. Hong[2], M. Stutts[3], S. L. Milgram[1]; [1]NHLBI, NIH, Bethesda, MD, [2]Membrane and Biology laboratory, Institute of Molecular and Cell Biology, Singapore, [3]Cystic Fibrosis Pulmonary Treatment and Research Center., UNC Chapel Hill, Chapel Hill, NC

The underlying defect in Cystic Fibrosis is the mutation of the Cystic Fibrosis transmembrane conductance regulator (CFTR) a cAMP-activated chloride channel, expressed at the apical surface of epithelia. The most prevalent disease-causing mutation, ΔF508, causes a biosynthetic defect in CFTR, resulting in the retention of the channel in the endoplasmic reticulum . Although various conditions promote ΔF508 CFTR exocytosis, restoring some function in vitro, the rescued protein is unstable at the cell surface and is rapidly internalized and degraded. Currently little is known about the mechanisms underlying CFTR internalization and recycling. Here we identify Sorting Nexin 27 (SNX27) as a novel regulator of this process. Using a proteomic approach we identified an interaction between the PDZ domain of SNX27 and the C-terminal PDZ-binding motif of CFTR. SNX27 localized within early and recycling endosomes through a mechanism requiring a functional PX domain, where it co-localized with internalized CFTR. Although SNX27 does not appear to effect the internalization of CFTR, interference of SNX27 function, via the expression of dominant-negative proteins or siRNA, caused a dramatic reduction of internalized CFTR recycling back to the cell surface. Our data provide further insight into the role of the C-terminus of CFTR in trafficking of the protein back to the cell surface and identify SNX27 as a novel regulator of this process.

817/B14

Ultrastructural and Biochemical Characterization of Trypanosoma Cruzi Reservosome Lipid Inclusions.

M. G. Pereira[1], C. Sant'Anna[1], E. Nakayasu[3], C. L. Alcântara[1], G. C. Atella[2], I. C. Almeida[3], W. De Souza[1], N. L. Cunha-e-Silva[1]; [1]Instituto de Biofisica Carlos Chagas Filho, Universidade Federal de Rio de Janeiro, Rio de Janeiro, Brazil, [2]Instituto de Bioquimica Medica, Universidade Federal de Rio de Janeiro, Rio de Janeiro, Brazil, [3]Biological Sciences, University of Texas at El Paso, El Paso, TX

Epimastigotes from Trypanosoma cruzi possess a remarkable ability to ingest different classes of macromolecules from medium, via cytostome or flagellar pocket, and accumulate them inside reservosomes until digestion. Reservosomes are acidic lysosome related organelles, which also store proteases, such as cruzipain and serine carboxypeptidase. They present an ATP binding cassette, TcABCA1, involved in cholesterol homeostasis, and store high amounts of neutral lipids, such as cholesteryl esters and cholesterol, probably as a result of low density lipoproteins (LDL) particles endocytosis (reviewed in Cunha-e-Silva et al., Parasitol. Res.99:325, 2006). Recently, our group have shown that inside reservosomes stored lipids can be observed as spherical lipid droplets or as rectangular inclusions surrounded by a phospholipid monolayer (Sant'Anna et al, Microsc. Res. Tech. 71:599, 2008), whose ultrastructural aspect is akin to that of lipid inclusions found in foam cells (Bobryshev 2006). Epimastigotes were cultivated in LIT medium supplemented with low (1- 5%) or high (20 - 50%) fetal calf serum. Electron microscopy analysis of thin sections of low serum epimastigotes showed reservosome fusion events and absence of lipid inclusions, while abundant sword-shaped lipid profiles were observed crossing the organelle in epimastigotes from high serum cultures. Furthermore, when high serum epimastigotes were incubated for three hours in serum free medium, lipid inclusions disappeared, suggesting that the parasite is able to metabolize these peculiar shaped lipids. We have isolated lipid inclusions from purified reservosome fractions. Electron microscopy showed an enriched fraction of both rectangular and spherical lipid inclusions. HPTLC first analysis revealed 36,8% cholesteryl esters, 13,8% cholesterol, 15,2% fatty acids, 17,8% triacylglycerols, 8% non determined lipid and 9,3% phospholipids. Nile Red stained intensely either reservosomes in situ or the highly concentrated lipid fraction. Mass spectrometry of isolated lipid fraction is in course, aiming to determine lipid composition and identify proteins associated to these structures.

818/B15

A Genomic Screen to Identify Mutants Hypersensitive to Hygromycin B Antibiotic in Baker's Yeast.

M. Banuelos, Q. Nguyen, D. Moreno, C. Aguilera, E. Gharakhanian; Biological Sciences, CSULB, Long Beach, CA

The vacuole of the fungus Saccharomyces cerevisiae (i.e. baker's yeast) has been an exemplary model for lysosomal studies because of its functional similarity to the lysosomes of higher eukaryotes. Our laboratory is interested in studying the vacuolar biogenesis, function and trafficking in yeast. Several mutants defective in such functions, have also been shown to be hypersensitive to Hygromycin B. With the objective of exploring the connection between Hygromycin B hypersensitivity and vacuolar defects, we performed a genomic screen for growth hypersensitivity to Hygromycin B (hhy mutants), using a non-essential deletion strain library covering 80% of the yeast genome. From this screen, fourteen potential HHY genes have been isolated. The uncovered mutants were characterized with respect to vacuole morphology, integrity of secretion, and growth sensitivity on various selective media. The results implicate defects in vacuole trafficking, morphology and/or function in the mutants.

819/B16

MCOLN3 is Involved in the Trafficking of Cargo Proteins along the Endo/lysosomal Compartment in the Retinal Pigment Epithelial Cell Line ARPE-19.

J. A. Martina, R. Puertollano; Laboratory of Cell Biology, NHLBI, NIH, Bethesda, MD

MCOLN3 is a Ca^{+2}-permable channel that belongs to the transient receptor potential (TRP) ion channel family and is regulated by pH. Defects in pigmentation and deafness are linked to mutations in the MCOLN3 gene that produce a gain-of-function in the varitint-waddler (Va) mice model. Alanine substitution by proline (A419P) in MCOLN3 results in a constitutively active channel, which loses regulation by pH and causes massive cell death. MCOLN3 channel remains active at the slightly acidic pH (~6.3) reported in several intracellular organelles like early and late endosomes but is inactive at a lower pH (~5.5) characteristic of lysosomes where active MCOLN1 localizes . The subcellular distribution of MCOLN3 is not well characterized and its role in protein trafficking is unknown. To address these issues we decided to study the subcellular localization of MCOLN3 in the retinal epithelial cell line ARPE19. Using immunofluorescence and confocal microscopy we found that heterologously expressed GFP-tagged-MCOLN3 (GFP-MCOLN3) localized to the plasma membrane and to the endo/lysosomal pathway in ARPE19 cells. Overexpressed GFP-MCOLN3 showed extensive co-localization with the early endosomal marker Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate), as well as with late endosomal/lysosomal markers such as LBPA (lysobisphosphatidic acid), Lamp1 and CD63. These results show a clear difference in the subcellular distribution between MCOLN3 and its relative MCOLN1, which is mostly lysosomal. Interestingly, over-expression of MCOLN3 effected the normal trafficking of endocytosed EGFR (Epidermal Growth Factor Receptor). Western blot analysis of ARPE cells over-expressing GFP-MCOLN3 showed a delayed degradation of EGFR. In addition, immunofluorescence studies in GFP-MCOLN3 expressing cells showed that fluorescent Alexa555-labeled EGF remained trapped at MCOLN3-positive endosomes 3h after internalization. In contrast no Alexa555-labeled EGF were detected after 3h in GFP expressing cells. All together, our data show that MCOLN3 is involved in protein trafficking along the endo/lysosomal pathway.

820/B17

Identification of the Penta-EF-Hand Protein ALG-2 as a Ca2+-Dependent Interactor of Mucolipin-1.

S. Vergarajauregui, R. Puertollano; Laboratory of Cell Biology, Cell Biology and Physiology Center, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD

Mucolipins constitute a family of ion channels with homology to the transient receptor potential family. Mutations in mucolipin-1 (MCOLN1) have been linked to mucolipidosis type IV (MLIV), a recessive lysosomal storage disease characterized by severe neurological and ophthalmologic abnormalities. In contrast with other lysosomal storage diseases, lysosomal hydrolases seem to be functional and correctly transported to lysosomes in MLIV, suggesting that the observed defect in lysosomal function may be due to alterations in trafficking along the lysosomal pathway. MCOLN1 contains sorting signals that regulate its transport to lysosomes, and undergoes posttranslational modifications such as palmitoylation and phosphorylation, that regulate its trafficking and channel activity. In addition, MCOLN1 is necessary for the efficient fusion of both autophagosomes and late endosomes with lysosomes. As these events are largely Ca2+ dependent, we hypothesized that MCOLN1 might have Ca2+ -dependent interactors that regulate its function. In order to test this hypothesis we performed pulldown experiments using the NH-terminal cytosolic tail of MCOLN1 fused to GST and HeLa cell extracts in the presence and absence of Ca2+. We found that MCOLN1 interacts with ALG2, a prototypic member of the penta-EF-hand family of proteins. We also observed that the interaction is highly dependent on Ca2+, as it was abrogated when HeLa extracts were treated with the Ca2+ chelator EGTA. In agreement with this, MCOLN1 did not bind the calcium-binding defective mutant ALG-2E47A/114A. The alternatively spliced isoform of ALG-2 (ΔGF) was not pulled down by MCOLN1 indicating that MCOLN1 belongs to the isoform-non-interactive group of ALG2 interactors, which include Alix, and TSG101. Moreover, immunoflourescence microscopic analysis in HeLa cells revealed that MCOLN1 and ALG-2 co-localize to enlarged endosomes induced by the over-expression of an ATPase-defective dominant negative form of Vps4B (GFP-Vps4BE235Q). Our findings suggest that the interaction between ALG-2 and MCOLN1 may regulate Ca2+-dependent events in the late endosomal-lysosomal pathway.

821/B18

Identification of Interactors of Mucolipins.

T. Lepow, T. Durns, E. McCarthy Campbell, H. Dang, H. Fares; Molecular and Cellular Biology, University of Arizona, Tucson, AZ

Lysosomes and pre-lysosomal compartments play an important role in the late endocytic pathway, notably in the targeting of endocytosed molecules to other compartments or in their degradation. These late compartments are very dynamic exhibiting homotypic and heterotypic fusions and reformation of compartments using mechanisms that are not completely understood. Mucolipin-1 is a transmembrane protein required for dynamic lysosomal trafficking events, and is hypothesized to function as a cation channel. In humans, a nonfunctional mucolipin-1 protein results in accumulation of large vacuoles eventually leading to neurodegeneration. This lysosomal storage disorder is known as Mucolipidosis Type IV. To better understand how mucolipin-1 and its close homolog mucolipin-2 function in lysosome biogenesis, we have performed a split-ubiquitin yeast two-hybrid screen. This split-ubiquitin system was designed for the identification of transmembrane protein interactions. Our results show interactions between the mucolipins and sorting nexins as well as homo and hetero-dimerization of the mucolipins. We are also using this system to perform screens for novel interactors of mucolipins with the aim of gaining a

more detailed understanding of lysosomal trafficking events. A more detailed understanding of these events may also prove valuable for developing treatments for patients with lysosomal storage disorders such as Mucolipidosis Type IV.

822/B19

The role of CUP-5 in the generation of lysosome-related organelles.

E. McCarthy Campbell, H. Fares; Molecular and Cellular Biology, University of Arizona, Tucson, AZ

Lysosome-related organelles share characteristics with lysosomes but carry out specific functions in specialized cells. In *C. elegans*, gut granules are intestine-specific organelles that accumulate birefringent material and have been identified as lysosome-related organelles. It is not completely understood how gut granules are formed, and how this process differs from that of lysosome differentiation. CUP-5 is a Transient Receptor Potential (TRP) protein in *C. elegans* that plays a role in endocytosis and lysosome biogenesis, and is homologous to mucolipin-I in mammals. In *cup-5* mutants, enlarged endo-lysosomal compartments are found in several cell types, including scavenger cells called ceolomocytes and developing intestinal cells. These compartments have characteristics of both late endosomes and lysosomes and cannot execute proper lysosomal degradation. Developing intestinal cells also accumulate enlarged gut granules. These *cup-5* defects are rescued by a mutation in MRP-4, an ABC transporter. The recent identification of MRP-4 as a player in gut granule differentiation suggests that CUP-5 may also play a role in gut granule differentiation. It is not clear yet whether CUP-5 plays a direct role in both lysosome and gut granule biogenesis, or whether a defect in lysosome biogenesis in *cup-5* mutants indirectly leads to a defect in gut granules. To test this, we are investigating the functions of CUP-5 in developing intestinal cells, a tissue that has morphologically and cell biologically well-defined lysosomes and gut granules. These experiments will determine whether CUP-5 is a central player in the biogenesis of post-endosomal compartments and will potentially give us a better understanding of the mechanisms that regulate the biogenesis of lysosome-related organelles.

823/B20

Autotaxin-mediated Control of Actin Dynamics through Rho-ROCK-LIM Kinase Pathway is Required for Endocytic Vesicle Fusion and Lysosome Biogenesis in Yolk Sac Visceral Endoderm Cells.

S. Koike[1], K. Keino-Masu[1], T. Ohto[1], F. Sugiyama[2], S. Takahashi[2], M. Masu[1]; [1]Molecular Neurobiology, Basic Med. Sci., University of Tsukuba, Tsukuba, Ibaraki, Japan, [2]Laboratory Animal Resource Center, University of Tsukuba, Tsukuba, Ibaraki, Japan

Autotaxin is an exoenzyme that produces lysophosphatidic acid (LPA), a lipid mediator possessing a wide variety of biological functions, including cell proliferation, migration and survival. Here we demonstrated that autotaxin$^{-/-}$ mice showed the reduction in the size of large lysosomal vesicles in yolk sac visceral endoderm (VE) cells at embryonic day 8.5 (E8.5). VE cells play a critical role in the maternofetal exchange of nutrients prior to establishment of a chorioallantoic placenta (~E9). Reflecting this function, VE cells have very large endosomal vesicles/lysosomes and vigorously endocytose maternal proteins. We hypothesized that some defects in endocytosis or vesicle trafficking might cause the abnormality of lysosome biogenesis in autotaxin$^{-/-}$ VE cells. Therefore, we examined trafficking of endocytic vesicles using time-lapse imaging after labeling them with fluorescent molecules. We showed that the transport from late endosomes to lysosomes was impaired in the autotaxin$^{-/-}$ VE cells because of the reduction of homotypic fusion of late endosome and heterotypic fusion between late endosomes and lysosomes. We also examined the downstream signaling pathways of autotaxin using *ex vivo* whole mouse embryo culture. We showed that inhibitors for LPA receptors (Ki16425), Rho (*Clostridium* C3 exoenzyme), ROCK (H1152, hydroxyfasudil, and Y-27632), and LIM kinases (S3 peptide) phenocopied the defects observed in autotaxin$^{-/-}$ VE cells. In addition, Alexa488-phalloidin staining revealed that actin filaments were associated with late endosomes in VE cells and actin polymerization were reduced in autotaxin$^{-/-}$ VE cells. Furthermore, actin stabilizing and depolymerising drugs phenocopied some aspects of autotaxin$^{-/-}$ VE cells. Taken together, actin assembly and disassembly regulated by autotaxin through the Rho/ROCK/LIM kinase pathway plays an important role in endocytic vesicle fusion and lysosome biogenesis.

824/B21

Identification and Function of Protein Storage Vacuoles in Mature Leaves.

M. Jang; Molecular and Life Science, Postech, Pohang, South Korea

The vacuole of Plant cells exists as multiple functionally distinct vacuolar compartments along each developmental stage. Plant vacuoles have divided into lytic vacuoles (LV) and protein storage vacuoles (PSV), largely. LVs function as acidic, hydrolytic, lysosome-like vacuoles in mature leaves and PSVs as neutral vacuoles in seeds. Recent studies have reported that LVs and PSVs co-exist in the Arabidopsis cotyledon. However, the evidences for multiple vacuoles within the same cell are insufficient and multivacuolar hypothesis in mature leaves have been in a debate. So we show clear evidences that plant cells have two distinct vacuoles in mature leaves and their distinguish functions. First, we examined whether various storage proteins such as β- conglycinin (soybean), phaseolin (soybean), chitinase (bean) transiently expressed in arabidopsis leaf protoplasts and made each transgenic plant. They all showed in same compartment like a disc pattern in the confocal microscopy and seemed to be targeted into the PSVs in the mature leaves. Next, we confirmed that these disc patterns did not aggregate by biochemical data. We will further study how disc patterns which are expressed in Arabidopsis mature leaves have a different role from developing seeds.

Metalloproteases (825 – 840)

825/B22

Atomic Force Microscopy Reveals a Collagenase-Dependent Local Softening Around Invasive Sites in a 3D Collagen Culture Model of Mammary Epithelial Branching Morphogenesis.

J. Alcaraz[1,2,3], H. Mori[1], D. Brownfield[1], C. Bustamante[2,1], M. Bissell[1]; [1]Life Sciences Division, Lawrence Berkeley National Laboratory,

Berkeley, CA, [2]Physics, and Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, [3]Unitat de Biofisica, Universitat de Barcelona, Barcelona, Spain

Cellular invasion through collagenous stroma is a hallmark of both branching organs and tumor progression. Using a 3D culture model of branching morphogenesis, we previously showed that invasion of non-malignant mammary epithelial cells through a collagen I gel was collagenase-dependent. However, it remains unknown how collagenase activity compromises spatially the mechanical properties of collagen I during this invasive process. We used atomic force microscopy to measure the stiffness of collagen I gels close to the edge of, or distal from (100 microns), branching sites in the 3D culture model. We found that collagen stiffness dropped nearly 50% at branching sites, and that this softening was abrogated using a broad spectrum inhibitor of matrix metalloproteinases (MMPs). Collagen softening at branching sites increased in cells overexpressing the collagenase MMP14, whereas it was inhibited in cells expressing shRNA against MMP14. Our results indicate that non-malignant cellular invasion through collagen I dramatically and locally downmodulates the rigidity of the collagen matrix and that this mechanical effect is largely mediated by MMP14. These findings help define the molecular basis underlying the mechanical extracellular alterations that occur during cellular invasion through collagenous stroma, an important process in both development and tumor progression.

826/B23

Expression Analysis of MMPs during Zebrafish Development.

N. D. Harris[1], J. Y. Keow[1], D. Li[2], C. Po[2], A. Hong[2], E. McKiel[1], B. D. Crawford[1]; [1]Biology, University of New Brunswick, Fredericton, NB, Canada, [2]Anaspec, Inc., San Jose, CA

Embryonic morphogenesis results from the remodeling of tissues at micro and mesoscopic scales. The degradation of the extracellular matrix (ECM) by matrix metalloproteinases (MMPs) is fundamental to this process, yet in vivo models for the study of MMP activity and regulation are limited. Recent advances have made the zebrafish an attractive system for studying this process, however, commercially available antibodies against MMPs have invariably been raised against mammalian homologues, and few are useful for studies using the zebrafish. The objective of this study was to develop effective immunological reagents for the study of MMPs in zebrafish. We tested a suite of twenty-nine antibodies directed against zebrafish homologues of these ECM remodeling enzymes, using western blots, and whole mount immunostaining of formaldehyde and TCA-fixed embryos. 17 of these antisera were found to be effective in one or more of these assays, and we report here on the the ontogeny of the antigens we were able to detect during the first 48 hours of embryonic development. These data will serve as a foundation for the use of zebrafish as a model system for investigating the regulation of ECM remodeling during development, regeneration, aging and pathological processes.

827/B24

ADAM15 SH3-Interactions Are Regulated by Alternative Splicing.

I. Kleino[1], A. J. Huovila[2], K. Saksela[1]; [1]Haartman institute, University of Helsinki, Helsinki, Finland, [2]Institute of Medical Technology, University of Tampere, Tampere, Finland

ADAM15 is a member of a adamalysin metalloproteinase and disintegrin protein family. Accumulating evidence associates it with a cancer progression as an adhesion protein and an ectodomain sheddase. ADAM15 cytosolic portion encoding exons are used alternatively yielding variability in ADAM15 cytosolic tail. ADAM15 isoforms have been shown to affect cell morphology and migration differently. Recent results have shown that cancer cell lines and tumors express distinct isoform patterns and that expression of some particular ADAM15 isoforms associate with relapse-free survival times. However, the details regarding how cytosolic tails change ADAM15 function is not well understood. In particular, coupling of ADAM15 to intracellular signaling and ADAM15 regulation are poorly understood. All except one ADAM15 isoform contain proline rich candidate SH3-binding motifs in cytosolic portion. Hence we decided to concentrate on ADAM15 isoform specific cytosolic SH3-interactions. Different ADAM15 cytosolic tails were used to screen the genome wide SH3-phage display library for potentially interacting SH3-proteins. The phage screens resulted many previously identified SH3-binding partners (Tks5, SNX9, Src, Yes, and Hck) and suggested some novel binders (e.g. SNX30 and Nephrocystin). The selected SH3 domains were used to probe peptide arrays containing individual ADAM15 SH3 target motifs. The solid phase assay results correlated well with the phage-library screening results, and allowed us to further map these interactions to defined regions among the multiple proline-rich clusters found in the intracellular tails of ADAM15 isoforms. The selected interactions were studied further in cell culture by pull-down assays with full length proteins and co-localization studies. Cell level studies were in agreement with the biochemical studies and indicated physical interaction of particular ADAM15 isoforms with certain SH3-domains also at cellular level.

828/B25

SirT1 Represses MMP13 Expression in Human Articular Chondrocytes.

E. Lee, V. Gagarina, M. Dvir-Ginzburg, D. Hall; CBOB, NIAMS, NIH, Bethesda, MD

Osteoarthritis (OA) is a degenerative joint disease of the articular cartilage resulting in the depletion of collagens and proteoglycans due in part to accelerated turnover and inadequate repair. Maintenance of healthy articular cartilage relies on the optimal expression of a number of unique extracellular matrix genes, such as aggrecan and collagen type II, by chondrocytes. MMP13, an enzyme for degradation of the extracellular matrix proteins including type II collagen, plays a critical role in bone remodeling and arthritis. SirT1 is an NAD-dependent histone deacetylase that regulates gene expression, differentiation, development and organism life span. The role of SirT1 involved in gene regulation in human chondrocytes has not been explored. Here we reported that MMP13 expression and activity are down-regulated by SirT1. Human chondrocytes (hChs) from the knees of osteoarthritic patients undergoing total knee arthroplasty were isolated and maintained in monolayer cultures. The SirT1-overexpressing cells showed the reduction of MMP13 protein by 2.5 fold. The activity of MMP13 was also decreased in SirT1-overexpressing cells by 2 times compared to control. These results indicate that MMP13 is suppressed by SirT1. These results were supported by (1) the knock-down of SirT1 which resulted in increase of MMP13 expression by 2 fold, and (2) the treatment of nicotinamide, a SirT1 inhibitor, which showed a significant increase of MMP13 mRNA levels by 3 fold in SirT1-overexpressing cells. Since SirT1 is believed to play an important role in enhancement of longevity, it was thought that SirT1 might regulate the expression of MMP13,

which plays a critical role in joint destruction in OA. The underlying mechanism by which SirT1 represses MMP13 is not well understood, however, we found that the level of Lef1, a known positive regulator of MMP13 expression, is also suppressed by SirT1. In addition, we also found several other MMP and ADAMTS proteins were also repressed by SirT1. Taken together, these data suggest that SirT1 regulates the expression of specific matrix proteins and plays a critical role in cartilage pathology.

829/B26

Asn362 Contributes to Zinc Binding by Leukotriene A4 Hydrolase.

M. Thompson, C. S. Thompson, R. L. Seipelt; Biology, Middle Tennessee State University, Murfreesboro, TN

Leukotrine A_4 hydrolase (LTA_4H) is a pro-inflammatory zinc metallopeptidase/epoxide hydrolase that contains an atom of zinc at the active site. This zinc ion is bound to two histidine residues and a glutamate residue at the active site center. In order to more fully understand how zinc is coordinated at the active site, conserved residues that were identified as possibly influencing zinc binding by the enzyme were altered by site-directed mutagenesis to study their functions. One of these residues, Asn^{362}, was mutated to Gln (N362Q), Glu (N362E), and Leu(N362L). The N362E and N362L mutants exhibited minimal catalytic activity and reduced zinc affinity, while the N362Q mutation exhibited reduced catalytic activity and slightly reduced zinc affinity, indicating that Asn^{362} likely influences the chemical environment of the nearby zinc coordinating residue Glu^{363} through a possible hydrogen bond interaction. Trp^{356}, situated at the edge of the active site cleft, appears to help maintain the conformation of the enzyme but does not contribute significantly to zinc binding. An understanding of catalysis by LTA_4H will help further the design of drugs that target the inflammatory process.

830/B27

Importance of Collagen Binding to Snake Venom Metalloproteinases-induced Hemorrhage.

C. Baldo[1], I. Tanjoni[1], R. Weinlich[4], D. S. Lopes[1], P. B. Clissa[1], G. P. Amarante-Mendes[4], N. Yamanouye[3], A. M. Chudzinky-Tavassi[2], C. Jamora[5], T. M. Zorn[4], A. M. Moura-da-Silva[1]; [1]Lab. de Imunopatologia, Instituto Butantan, São Paulo, Brazil, [2]Lab. de Bioquímica, Instituto Butantan, São Paulo, Brazil, [3]Lab. de Farmacologia, Instituto Butantan, São Paulo, Brazil, [4]Instituto de Ciências Biomédicas, Universidade de São Paulo, São Paulo, Brazil, [5]Biology, University of California, San Diego, CA

Hemorrhage, an important symptom of snake bite victims, is mostly due to the action of Snake Venom Metalloproteinases (SVMPs) and is directly related to their catalytic action towards plasma and extracellular matrix (ECM) proteins. However, it is unknown why only certain members of SVMPs class P-III induce strong hemorrhage and some P-I SVMPs exhibit lower activity. This suggests that other mechanisms besides catalysis may affect the integrity of endothelium allowing its disruption and bleeding. In order to identify the mechanisms involved in SVMP-induced hemorrhage, we compared the action of jararhagin (highly hemorrhagic P-III) and BnP1 (weakly hemorrhagic P-I) on endothelial cells, ECM and plasmatic proteins and mouse skin model. Jararhagin and BnP1 equally induced endothelial cell damage characterized by HUVEC-specific alterations in F-actin cystokeleton, cell detachment and decrease in cell viability followed by apoptosis. Both toxins degraded fibrinogen, fibrin, and collagen I. However, binding to plasma and ECM proteins was restricted to jararhagin/collagen I and IV. Berythractivase, a non-hemorrhagic P-III SVMP, was used as a control and also failed to bind to collagen. In mouse model, epidermis and basement membrane were not affected by injection of jararhagin and BnP1. However, 1 hour after injection of 1mg jararhagin, extensive breakdown of dermal collagen fibers was observed in the histological sections. This effect was not observed in mice injected with control or the same dose of BnP1. The fibrillar collagen distribution was affected by both toxins, but only jararhagin induced an evident degradation of collagen fibers in hypodermis, where most of the hemorrhagic lesion was observed. According to these data, catalysis of plasma/ECM proteins and endothelial cell damage were similarly induced by hemorrhagic and non-hemorrhagic SVMPs and hemorrhage was correlated with binding to collagen and disruption of its fibers. Thus, we suggest that binding to collagen I lead to a concentration of the enzyme on hypodermis, and consequent damage of its collagen-rich ECM, weakening of the capillary vessel and resulting in the strong local hemorrhagic activity of P-III SVMPs. Supported by FAPESP and CNPq.

831/B28

Ursolic Acid Inhibits Matrix Metalloproteinase Activity in DU145 Human Prostate Cancer Cells.

B. E. Scott[1], C. Neto[3], K. Gottschall-Pass[2], R. Hurta[1]; [1]Department of Biology, U.P.E.I., Charlottetown, PE, Canada, [2]Department of Family & Nutritional Sciences, U.P.E.I., Charlottetown, PE, Canada, [3]Department of Chemistry & Biochemistry, UMass-Dartmouth, North Dartmouth, MA

Matrix metalloproteinases (MMPs) are a family of proteases often over-expressed in cancer cells and their over-expression is associated with increased invasiveness and increased malignant potential. Ursolic acid (UA) is a triterpene found in many fruits including the American cranberry (Vaccinium macrocarpon) . UA treatment of DU145 human prostate cancer cells resulted in a dose dependent decrease in MMP-9 activity levels and an increase in MMP-2 activity levels (assessed by zymography). Treatment of DU145 prostate cancer cells with UA (10 uM and 50 uM) resulted in induction of TIMP-1 / TIMP-2 expression and RECK expression [TIMPs and RECK are inhibitors of MMPs] . EMMPRIN [an activator of MMPs' expression] was inhibited by UA. These results suggest that UA can affect the expression and regulation of MMPs' activity in DU145 prostate cancer cells. Studies are ongoing to elucidate the signal transduction mechanisms involved. [National Cancer Institute of Canada, Canadian Cancer Society, P.E.I. Health Research Program, Atlantic Canada Opportunities Agency-Atlantic Innovation Fund - funded]

832/B29

MT1-MMP Induces Cancer Cell Epithelial-Mesenchymal Transition by Upregulating Wnt-5a and Inducing ROS.

H. Nguyen[1,2], S. Zucker[2,1], J. Cao[1,2]; [1]Medicine, Hematology Division, State University of New York at Stony Brook, Stony Brook, NY, [2]Medicine, VA Medical Center, Northport, NY

BACKGROUND: A key step in the evolution of cancer from an early stage to an invasive, metastatic phenotype is thought to involve the epithelial-to-mesenchymal transition (EMT) of cancer cells. This process involves the cancer cell's epithelial phenotype and its acquisition of mesenchymal or fibroblastic characteristics. Expression of the membrane-bound matrix metalloproteinase MT1-MMP has been shown to be

associated with tumor invasion. We have previously shown that overexpression of MT1-MMP induces morphologic changes in the prostate cancer cell line LNCaP that are characteristic of EMT. Moreover, we showed that MT1-MMP overexpression upregulates Wnt-5a, a protein which has been implicated in cancer cell proliferation, invasion, and motility. OBJECTIVE: To study the mechanism by which MT1-MMP expression induces EMT. METHODS and RESULTS: Here, we report that overexpression of MT1-MMP enhances LNCaP cell proliferation, and shRNA knockdown of Wnt-5a in these MT1-MMP overexpressing cells dramatically decreased the cell proliferation rate to even below that of control mock-transfected cells. MT1-MMP overexpression also enhanced growth in mouse xenograft tumors from LNCaP cells, and shRNA knockdown of Wnt-5a, likewise, repressed tumor growth rate to below that of tumors from control cells. MT1-MMP enhanced intracellular reactive oxygen species (ROS); inhibition of MT1-MMP's enzymatic activity with the MMP inhibitor, BB3103, decreased ROS to that of control mock-transfected cells, suggesting that MT1-MMP induces ROS via its extracellular enzymatic activity. Overexpression of Wnt-5a also enhanced ROS in LNCaP cells, suggesting that one mechanism by which MT1-MMP induces ROS is via upregulation of Wnt-5a. The generation of intracellular ROS has been shown to be an important for mediating expression of E-cadherin and integrin, which play key roles in EMT and cell migration. CONCLUSION: We provide evidence of a mechanism by which MT1-MMP mediates EMT and tumor cell invasion, by upregulating Wnt-5a, which enhances cell proliferation and induces ROS.

833/B30

Downregulation of Matrix Metalloproteinase-9 by IFN-β Requires HDAC1 Recruitment to the Promoter via the Proximal AP-1 Site.

M. Mittelstadt[1], A. Kaufman[1], C. V. Patel[2], R. C. Patel[1]; [1]Biological Sciences, University of South Carolina, Columbia, SC, [2]Cell, Developmental Biology, and Anatomy, University of South Carolina, Columbia, SC

Matrix metalloproteinase-9 (MMP-9) is one of the secreted zinc dependent metalloproteinases that degrades components of the extracellular matrix. Regulation of MMP-9 is essential for many developmental processes including angiogenesis, embryonic development and wound healing. Enhanced expression of MMP-9 is involved in pathological processes such as metastasis, arthritis, multiple sclerosis, and asthma. A complete understanding of the transcriptional regulation of MMP-9 could allow for novel therapeutic strategies. Although it has been widely noted that interferon-β (IFN-β) downregulates both the basal and phorbol 12-myristate 13-acetate (PMA)-induced MMP-9 expression at the transcriptional level, the molecular mechanism of this repression has not been completely elucidated. In the present study we identify a novel mechanism for downregulation of MMP-9 transcription by IFN-β in HT1080 fibrosarcoma cells. Using reporter assays with promoter deletion constructs we showed that IFN-β's inhibitory effects require the proximal AP-1 site. Chromatin immunoprecipitation studies indicated that IFN-β increases histone deacetylase (HDAC)-1 recruitment to the MMP-9 promoter, while reducing NF-κB recruitment. In accordance with this, IFN-β's repressive actions were abrogated in the presence of a type 1 HDAC inhibitor, Trichostatin A (TSA). Analysis of the promoter deletion series and reporter assays indicated that the inhibitory effect of TSA on IFN-β's repressive actions requires the proximal AP-1 site. An artificial firefly luciferase reporter construct with a minimal promoter and AP-1 site from MMP-9 mimics both the IFN-mediated repression and TSA-mediated abrogation of IFN's repressive effects, thereby demonstrating the important role of the proximal AP-1 site. Loss of a functional AP-1 site from the MMP-9 promoter by a point mutation results in markedly reduced ability of IFN-β to repress the MMP-9 transcription. These results further establish that the proximal AP-1 site is essential for HDAC1 recruitment to the MMP-9 promoter in response to IFN-β. A possible involvement of post-translational modifications on AP-1 in response to IFN-β in mediating HDAC1 recruitment will be investigated in future studies.

834/B31

Novel Lminin5 γ2-chain Fragments Function as Motility Factors for Limbal Epithelial Cell Outgrowth on Amniotic Membrane.

C. Sun[1,2], C. Cheng[3], H. Hsieh[4], C. Yang[3]; [1]Ophthalmology, Chang Gung Memorial Hospital, Keelung, Taiwan, [2]Chinese Medicine, Chang Gung University, Taoyuan, Taiwan, [3]Pharmacology, Chang Gung University, Taoyuan, Taiwan, [4]Nursing, Chang Gung Institute of Technology, Taoyuan, Taiwan

Matrix metalloproteases (MMPs)-mediated extracellular matrix (ECM) degradation potentially releases cryptic motility factors involving in somatic stem cell migration and epithelial outgrowth. MMP-9 was up-regulated in limbal epithelial cells cultivated on amniotic membrane (AM). Here, we investigated whether plasminogen activator (PA)/plasmin regulated MMP-9 activity in this model implicated in the processing of laminin 5 (Ln5), a component of amniotic basement membrane. The results revealed that activity and expression of uPA were up-regulated in intact AM-expanded limbal cultures. Evidence showing that PA/plasmin regulated MMP-9 expression was from (1) attenuation of MMP-9 activity by uPA inhibitor, B428 and (2) enhancing MMP-9 activity by exogenously added or pre-incubated plasmin. An interesting proteolytic fragment of Ln5γ2-chain was suppressed by GM 6001, MMP-2/-9 inhibitor, or B428, implicated that Ln5 γ2-chain was processed by uPA/MMP-9. The extent of limbal outgrowth was also retarded by B428. These results suggested that MMP-9 activity was up-regulated by PA/plasmin which processed Ln5 γ2-chain to facilitate limbal outgrowth on intact AM.

835/B32

α4β1 Integrin and CD44v Constitute a Novel Docking Complex for Gelatinase B/MMP-9 in Leukemic B Cells.

J. Redondo-Muñoz[1], E. Ugarte-Berzal[1], J. A. García-Marco[2], M. Hernández del Cerro[1], P. E. Van den Steen[3], G. Opdenakker[3], M. Terol[4], A. Garcia-Pardo[1]; [1]Cellular and Molecular Physiopathology, Centro de Investigaciones Biológicas, CSIC, Madrid, Spain, [2]Hematología, Hospital Puerta de Hierro, Madrid, Spain, [3]Immunobiology, Rega Institute for Medical Research, Leuven, Belgium, [4]Hematología y Medicina Oncológica, Hospital Clínico, Valencia, Spain

As B-cell chronic lymphocytic leukemia (B-CLL) progresses, malignant cells extravasate and infiltrate lymphoid tissues. Several molecules, including gelatinase B/MMP-9, contribute to these processes. Although mainly a secreted protease, some MMP-9 is present at the B-CLL cell surface and the function, mode of anchoring and interactions of this MMP-9 are unknown. Here we show that anti-MMP-9 antibodies immunoprecipitated a 190 kDa CD44v isoform and α4β1 integrin from B-CLL cells, but not from normal B cells. Function-blocking antibodies to α4β1 or CD44 or transfection with specific siRNAs, decreased cell-associated proMMP-9 and increased the secreted form. B-CLL cells attached to and bound pro- and active MMP-9 and this was inhibited by blocking the expression or function of α4β1 or CD44. The MMP-9 hemopexin domain was critical in these interactions. α4β1 and 190 kDa CD44v (but not CD44H) formed a complex at the cell

surface, since they both co-immunoprecipitated with anti-α4, anti-β1 or anti-CD44 antibodies. Immunofluorescence analyses confirmed that α4β1 and CD44v colocalized with MMP-9. Binding of proMMP-9 inhibited B-CLL cell migration and this required MMP-9 proteolytic activity. Thus, we have identified α4β1 and CD44v as a novel proMMP-9 cell surface docking complex and we show that cell-associated MMP-9 may regulate B-CLL cell migration and arrest. Supported by grant PI060400 from the Ministerio de Sanidad y Consumo, and by the Fundación de Investigación Médica Mutua Madrileña.

836/B33

Methanol Extract of Elaeagnus Glabra, a Korean Medicinal Plant, Inhibits HT1080 Tumor Cell Invasion.

L. Li[1], I. Baek[1], S. Choi[2], B. Shin[1]; [1]Microbiology, Chonnam National University, Gwangju, South Korea, [2]Biomedical Sciences, Chonnam National University, Gwangju, South Korea

Elaeagnus glabra (Thunb.), an evergreen shrub with alternate leaves, has been used as a medicinal plant in Korea. Since many plant-derived molecules have inhibitory effects on tumor cell invasion, primarily via suppression of matrix metalloproteinases (MMPs) activities, we investigated the effect of the methanol extract of E. glabra on tumor cell invasion. The invasiveness of HT1080 human fibrosarcoma cells were reduced in a dose-dependent manner following 24-hr treatment of up to 200 µg/ml of the E. glabra extract, at which concentration no cytotoxicity occurred. Furthermore, gelatinolytic activities, and the protein and mRNA levels of MMP-2 and MMP-9 were also suppressed with increasing concentrations of the extract. Given that MMP-2 and MMP-9 are instrumental in tumor cell invasion, it is very likely that the reduction in tumor cell invasion by E. glabra extract is a consequence, at least in part, of suppressed expression of both MMP-2 and MMP-9. Isolation of a molecule(s) responsible for the extract inhibition of tumor cell invasion would pave the way for the development of a new generation of metastasis inhibitors.

837/B34

MMP9 Overexpression Correlates with Increased Myogenic Cell Migration In Vitro And Cell Dispersion In Vivo.

J. Morgan[2], P. Bausero[3], A. Houssaïni[1], A. Rouche[1], M. Fiszman[1], H. Alameddine[1]; [1]INSERM U582, AIM, Institut de Myologie, Paris, France, [2]The Dubowitz Neuromuscular Centre, UCL Institute of Child Health, London, United Kingdom, [3]UMR 7079 de Physiologie et Physiopathologie, Université Pierre et Marie Curie, Paris, France

The objective of this study was to investigate the role played by Mmp2 and Mmp9 or their resulting hydrolytic activity in myogenic cell migration and/or fusion. This was explored using two subtypes of C2C12 cells. MMPs expression pattern and overall gelatinolytic activity were determined in both cell types and were correlated to cell differentiation and migration in vitro and in vivo. Timp1 and Timp2 regulation was also monitored to assess the importance of MMPs/TIMPs balance in cell dispersion. Our results showed that the first identified cell type, C2M9, produced moderate amounts of Mmp2 and high levels of Mmp9 whereas the second, C2F, secreted important levels of Mmp2 but faint levels of Mmp9 resulting in a higher overall gelatinolytic activity in C2F cells. Interestingly, Timp1 and Timp2 were differentially expressed and regulated. C2F myoblasts expressed higher Timp1 mRNA levels in comparison to C2M9 cells. These levels were increased 24 hours after shifting C2F cells into differentiation medium but were downregulated in C2M9 myotubes. Timp2 mRNA and protein levels were not significantly regulated with cell fusion in both cell types. In vitro these cells exhibited different migratory capacities. C2M9 cells migrated better than C2F suggesting that Mmp9 over-expression could be responsible for increased migration. C2F cell migration assays carried in presence of C2M9 cells, of medium conditioned by C2M9 and finally, of added Mmp9, all resulted in increased migration. To the contrary, Mmp9 inhibition reduced C2M9 cell migration in a dose dependent manner. C2M9 also exhibited higher fusion capacities than C2F in vitro. Both characteristics were confirmed by cell grafting into nude-mdx muscles. In comparison to C2F cells, C2M9 cell grafting gave rise to more tissue of donor origin, increased muscle wet weights and number of dystrophin-positive muscle fibers indicating a better dispersion and fusion of C2M9 cells with host myofibers. These data underscore the importance of monitoring MMPs and TIMPs expression and levels in myoblasts dedicated to cell therapy and open new possibilities for myogenic cell engineering expected to impact on the efficiency of myogenic cell dispersion and gene complementation.

838/B35

In Vitro Effects of Tannic Acid, Gallic Acid and Ellagic Acid on Matrix Metalloproteinase Activity in Human Breast Cancer Cells.

B. A. Deziel, M. A. Drake, R. Hurta; Biology, University of Prince Edward Island, Charlottetown, PE, Canada

The consumption of phytochemicals derived from plant sources has been shown to decrease cancer risk. Breast cancer is the most common neoplasm in women worldwide. The present study assessed the ability of 3 phytochemicals to regulate an aspect of breast cancer behaviour. MDA-MB-231 cells were treated with tannic acid [TA], gallic acid [GA] and ellagic acid [EA] [10, 50 , 100 uM] for 6 and 24 hours, respectively and MMP activity was assessed by zymography. Post 24 hours treatment, a dose dependent decrease in MMP-9 activity occurred in response to TA, GA, and EA. A dose dependent decrease in MMP-2 activity occurred only in response to TA. The expression of modulators of MMP activity / expression , namely, TIMPs, EMMPRIN, and RECK , was also evaluated using Western blot analysis. GA increased RECK and TIMP-1 expression and had no apparent effect on TIMP-2 expression with a possible activation of EMMPRIN expression. TA increased RECK and TIMP-1 expression and decreased EMMPRIN expression and did not affect TIMP-2 levels. EA increased TIMP-1 levels and apparently did not affect EMMPRIN levels. The nature of the specific signal transduction pathways involved in the EA, TA, and GA mediated regulation of MMPs' activity in these breast cancer cells are ongoing.

839/B36

LPA Stimulation of Cell Surface-Associated Proteases in Ovarian Cancer Cells.

K. Chaffin[1], T. McKenna[1], K. Huegel[1], Y. Liu[2], S. Stack[2], S. M. Ellerbroek[1]; [1]Chemistry, Wartburg College, Waverly, IA, [2]Pathology and Anatomical Sciences, University of Missouri School of Medicine, Columbia, MO

Lysophosphatidic acid (LPA) contributions to ovarian cancer are postulated to involve stimulation of cell surface-associated proteolysis, therefore we analyzed the effects of LPA on the expression and cell surface association of two proteolytic enzymes linked with ovarian cancer progression. LPA significantly increased the density of OVCA 429 and OVCA 433 ovarian cancer cells at or below concentrations

normally found in ascites fluids (0.1 uM), whereas its stimulation of uPA activity in culture medium required the elevated concentrations (20-80 uM) often found in the ascites of ovarian cancer patients. Inhibitor studies demonstrated that OVCA 433, but not OVCA 429, cellular release of uPA activity required p42/44 MAPK signaling. Conversely, inhibition of p38 MAPK signaling in OVCA 433 cells stimulated the release of uPA activity in a dose-dependent fashion. Using a cell surface biotinylation and monomeric avidin pulldown approach, we found that LPA treatment (80 uM) significantly stimulated the binding of uPA activity to the surface of both OVCA 429 and OVCA 433 cells. Acid washing of cells removed cell surface-associated uPA activity. LPA treatment increased cellular release of MMP-9 over MMP-2 in conditioned media as measured by zymography and ELISA (3-5 fold), but did not stimulate detectable binding of MMP-9 to the cell surface. LPA stimulation of MMP-9 expression was dose-dependent and required p38 MAPK and PI3K signaling. Overexpression of MMP-9 by control cells was sufficient to drive cell surface association of a proteolytically processed MMP-9 species not observed in conditioned media. LPA stimulation of matrigel invasion (25-30 fold) by OVCA cells was blocked by both MMP (70-75%) and uPA (75-85%) inhibitors. These results suggest that an increased expression of MMP-9 and cell surface binding of uPA activity are involved in LPA-driven ovarian cancer invasion. Future studies will continue to test this hypothesis and specifically focus on identifying a mechanism(s) through which LPA promotes binding of uPA activity to ovarian cancer cell surfaces.

840/B37

MMP-1 Promoter SNPs Increase the Risk to Idiopathic Pulmonary Fibrosis (IPF).

A. Pardo[1], M. Checa[1, 2], V. Ruiz[2], M. Montaño[2], M. Selman[2]; [1]Facultad de Ciencias, Universidad Nacional Autonoma de Mexico, Mexico DF, Mexico, [2]Instituto Nacional de Enfermedades Respiratorias Ismael Cosio Villegas, Mexico DF, Mexico

The pathogenic mechanisms implicated in the development of IPF are presently unknown. We have demonstrated that, despite progressive scarring, MMP1 is strongly upregulated at the gene and protein levels suggesting that it may play a role in the aberrant lung remodeling that characterizes IPF. Recent evidence suggests that some MMP1 promoter single nucleotide polymorphisms (SNPs) affect the gene transcription. Particularly, the 2G allele of the MMP1-1607 1G/2G polymorphism has been associated with higher MMP-1 expression levels. The aim of this study was to evaluate this SNP in a cohort of 130 IPF patients and 305 controls with similar ethnic background. A significant increased frequency of the 2G/2G genotype was observed in the IPF patients (0.63 versus 0.49; p = 0.008; OR, 1.7; 95% CI, 1.15-2.79) suggesting that this SNP may influence the development of the disease. Then, we sequenced the promoter of the MMP1 and found a new polymorphism at the position -735 (G/T) that creates a potential binding site for transcription factors of the AP-1 family which could modulate the transcriptional activity of MMP1. When we examined the frequency of this polymorphism in our IPF and control cohorts, no differences were found. However, when we stratified our cohorts by cigarette smoking status (former/current versus non-smokers) we found that the TT genotype was significantly increased in smoker IPF patients compared with smoker controls (OR=2.3; CI 1.15-4.97; p=0.03). These data indicate that this new MMP1 gene variation may be a genetic factor contributing to the development of the disease in smokers.
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Tight Junctions (841 – 857)

841/B38

Regulation of EMT in Epithelial Cells by Bves, a Novel Adhesion Molecule.

M. S. Chang[1,2], S. H. Presley[1], J. Yang[1]; [1]Ophthalmology, Vanderbilt University, Nashville, TN, [2]Biomedical Engineering, Vanderbilt University, Nashville, TN

Objective: Bves (blood vessel epicardial substance) is a novel adhesion molecule isolated from developing cardiac tissues and observed to play a role organogenesis by regulating epithelial cell-cell contact formation and cellular migration. We have recently established that Bves regulates tight junction (TJ) formation. These observations led to the hypothesis that a molecular basis of Bves' biologic effects is through TJ associated signaling. Methods: Stably transfected cells overexpressing a full length Bves (WTX) and dominant negative truncated Bves (DN) were generated from a human corneal epithelial cell line (HCE). Isolates were analyzed for alterations in cytoskeleton, TJ formation, cell growth characteristics (soft agar assay and growth rate), cell motility, and cell morphology. In addition, co-immunoprecipitation studies were performed to determine Bves and DN-Bves interactions with TJ proteins. Finally, the state of Rho activation was determined. Results: The DN cells exhibited impaired monolayer formation with loss of epithelial morphology. In addition, DN cells exhibited other mesenchymal features such as expression of vimentin, increased motility, formation of colonies within soft agar, and decrease of levels of TJ proteins. These findings suggest that DN cells have undergone an epithelial-mesenchymal transition. In contrast, WTX cells retained their epithelial features. The levels of TJ proteins correlated with Bves level as indicated by measuring the trans-epithelial electrical resistance and by Western blotting for TJ proteins. Further, Bves co-immunoprecipitated with ZO-1 and with GEF-H1, an activator of RhoA, while DN Bves does not. GEF-H1 is known to be sequestered by TJs. Consistent with this DN cells exhibited an increase in RhoA activation, as indicated by a 2 fold increase in FRET compared to HCE. Conclusion: The regulation of the epithelial/mesenchymal phenotype with subsequent effects on cellular motility and cell-cell contact formation are the proposed basis for Bves role in organogenesis. Since TJs alters Rho signaling by sequestering GEF-H1, a molecular mechanism for effects of Bves on epithelial/mesenchymal phenotypes may be through modulation of Rho signaling.

842/B39

Treatment of Sertoli Cells with Bisphenol A (BPA) as a Novel in vitro Model for Studying Blood-Testis Barrier Dynamics.

W. Li[2], W. Lee[2], C. Cheng[1]; [1]Center for Biomedical Research, Population Council, New York, NY, [2]School of Biological Sciences, The University of Hong Kong, Hong Kong SAR, China

BPA is an estrogenic environmental toxicant found in many consumer products. Due to a constant exposure to this endocrine disruptor in humans, concerns were raised about its possible reproductive toxicity. Since most toxicants (e.g., cadmium, glycerol) are known to disrupt spermatogenesis by affecting blood-testis barrier (BTB) function and germ cell adhesion, the effects of BPA on junction integrity in the

seminiferous epithelium and thus spermatogenesis in adult rats were assessed. Rats (~300 gm b.w.) were treated daily with acute doses of BPA (0.02, 2, 10, or 50 mg/kg b.w.) for 5-6 days. No significant differences were detected at 1- to 11-weeks between treatment and control groups regarding body weight, testis weight, and steady-state levels (as assessed by immunoblotting) and localization of several cell junction proteins. When the status of spermatogenesis was assessed by histological analysis, however, a small and statistically insignificant percentage of seminiferous tubules displayed signs of germ cell loss in the 10 and 50 mg/kg treatment groups vs. controls. When a fluorescent tracer, inulin-FITC (Mr ~4.5 kDa), was administered via the jugular vein, it failed to penetrate the BTB as in control rats, indicating the barrier was not compromised in the treatment group. It is probable that the low bioavailability of free BPA in the systemic circulation failed to compromise spermatogenesis. However, using primary Sertoli cells cultured *in vitro* with a functional BTB that mimicked the BTB *in vivo*, BPA caused a reversible decline in tight junction integrity at a non-cytotoxic dose of 200 μM, but not at 40 μM. An induction of the ERK pathway was observed, along with a significant decline in the protein levels of tight junction (occludin and JAM-A), basal ES (N-cadherin), hemidesmosome (integrin β1), gap junction (connexin 43), and adaptors (α- and β-catenin). Studies by immunofluorescence microscopy and cell surface protein biotinylation techniques also illustrated declining levels of occludin and connexin 43 at the cell-cell interface following BPA treatment. In summary, BPA perturbs Sertoli cell BTB integrity reversibly and dose-dependently, which can serve as a novel model for studying BTB dynamics.

843/B40

Claudins 1, 3, 4 and 7 are Essential in Maintaining the Barrier Function of Tight Junctions in the Human Blood-Epididymal Barrier.

E. Dubé[1], J. Dufresne[1], l. hermo[2], P. Chan[3], D. Cyr[1,2]; [1]INRS-Institut Armand-Frappier, Laval, QC, Canada, [2]Department of Anatomy and Cell Biology, McGill University, Montreal, QC, Canada, [3]Department of Urology, Royal Victoria Hospital, Montreal, QC, Canada

The microenvironnement of the epididymal lumen is important for the transport, maturation and storage of spermatozoa. This environnement is created by the blood-epididymal barrier which is formed by apical tight junctions (TJ) between adjacent principal and between principal and clear cells that line the epididymal lumen. Both human and rat epididymal TJs are comprised of occludin and multiple claudins (Cldns). There is limited information on the cellular signalling pathways that regulate epididymal tight junctional proteins or on the role of each Cldn within the epididymal junctional complex. Using novel human epididymal epithelial cell lines derived from fertile (FHCE1) and infertile (IHCE1) patients, our objectives were to determine the signalling pathways implicated in the formation of epididymal TJs and to assess the role of certain Cldns in maintaining the integrity of the human epididymal epithelium. Using a Ca2+ switch model, we were able to induce the formation of TJs between FHCE1 cells but not in IHCE1 cells, as determined by measuring the transepithelial resistance (TER) across the cells. This is consistent with the fact that IHCE1 cells expressed lower levels of several genes encoding junctional proteins, including CDH1, CDH2, Cldns 1, 3, 4, 7 and 8. Using oligonucleotide microarrays we compared gene expression profiles between FHCE1 and IHCE1 in the presence/absence of Ca2+ during TJ activation. Our results indicated that several genes that are known be implicated in TJ formation were expressed at higher levels in FHCE1 cells that form TJs, including CDC42, PKA, RAB13 and PKCz. To assess the role of Cldns in epididymal TJs, an siRNA approach was used with FHCE1 cells. Results indicate that TER was lost when the expression of either Cldns 1, 3, 4 or 7 was decreased but was unaltered by inhibition of Cldns 2 and 8. These results suggest that Cldns 1, 3, 4 and 7 are essential for barrier function in human epididymal TJs and that the regulation of these TJs depends on the activation of specific intracellular signalling pathways. Supported by CIHR, FRSQ, and Armand-Frappier Foundation.

844/B41

The Tight Junction Zonula Occludens Proteins Inhibit Cyclin D1/Cdk4 Dependent Cell Proliferation Through Membrane Sequestration.

C. T. Capaldo, A. Nusrat; Pathology, Emory University, Atlanta, GA

Epithelial tight junctions function to seal adjacent cells, forming a barrier to paracellular diffusion of fluid and solutes. Recently, tight junctions have also been shown to regulate cell cycle progression through the sequestration of transcription factors and signaling molecules at tight junction contacts. It has been reported that the cytoplasmic tight junction plaque protein, ZO-1 (Zonula Occludens-1) mediates this effect by regulating Cyclin D1 gene expression and signaling, in a cell density dependent manner. In order to further explore the mechanisms through which ZO family proteins (ZO-1, ZO-2, ZO-3) regulate cell cycle progression, we investigated the relationship between Cyclin D1/Cdk4 (Cyclin dependent kinase) and tight junction protein complexes. We now report that Cyclin D1and Cdk4 co-localize with ZO-1 and ZO-2 at tight junction contacts, in both human and murine colonic epithelial cells. Biochemical fractionation and density gradient analysis of a model intestinal epithelial cell line, SKCO15, confirmed membrane association of Cyclin D1-Cdk 4 with tight junction scaffolding complexes. Importantly, a proportional increase in Cyclin D1 protein was identified in membrane enriched fractions from confluent epithelial monolayers, when compared with low confluence cultures. Consistent with these observations, Cyclin D1 and ZO family proteins co-immunoprecipitated from the membrane enriched fractions. Interestingly, amino acid sequence analysis of Cyclin D1 revealed an evolutionarily conserved, c-terminal Class II PDZ (PSD-95, Dlg, ZO-1) binding motif. GFP-Cyclin D1 fusion proteins lacking this motif exhibit altered sub-cellular localization and increased its accumulation in the nucleus. Additionally, overexpression of this mutant enhanced the ability of Cyclin D1 to promote cell cycle progression. These data demonstrate that Cyclin D1 function can be regulated by its interactions with ZO plaque proteins, and represents a novel mechanism of cell cycle regulation, through cell density dependent accumulation of Cyclin D1 at intercellular tight junctions.

845/B42

The E3 Ubiquitin Ligase LNX1p80 Downregulates Claudins from Tight Junctions in MDCK Cells.

S. Takahashi[1], N. Iwamoto[1,2], H. Sasaki[3], M. Ohashi[4], Y. Oda[2], S. Tsukita[1], M. Furuse[2]; [1]Department of Cell Biology, Kyoto University Faculty of Medicine, Kyoto, Japan, [2]Department of Physiology and Cell Biology, Kobe University Graduate School of Medicine, Kobe, Japan, [3]Department of Molecular Cell Biology, Institute of DNA Medicine, The Jikei University School of Medicine, Tokyo, Japan, [4]Laboratory of Nano-Structure Physiology, Okazaki Institute for Integrative Bioscience, National Institutes of Natural Sciences, Okazaki, Japan

The structural continuity of tight junctions (TJs) is consistently maintained even when epithelial cells divide and move within the cellular sheet. This process is associated with dynamic remodeling of TJs by coordinated destruction and generation of claudin-based TJ strands, but the molecular mechanism behind the regulated turnover of TJs remains largely unknown. In this study, we identified the p80 isoform of the E3 ubiquitin ligase Ligand-of-Numb protein-X 1 (LNX1p80) as a protein binding to claudin-1. Interestingly, the concentration of claudins in TJs was remarkably reduced when LNX1p80 was overexpressed in MDCK cells, and there was a reduction not only in the number of TJ strands but also in the amount of detergent-insoluble caludins. We also found that LNX1p80 promoted poly-ubiquitination of claudins. This ubiquitination is dependent on its RING-finger domain and is not mediated by lysine 48 of ubiquitin, which is used for protein degradation by proteasome. Furthermore, LNX1p80 was often colocalized with claudins in vesicular structures containing markers for late endosomes and lysosomes. These findings suggest that LNX1p80 is involved in the ubiquitination, endocytosis, and lysosomal degradation of claudins, and that the turnover of TJs is regulated by ubiquitination.

846/B43

A Claudin Modulator Is a Promising Candidate for Drug Absorption.

M. Kondoh[1], A. Takahashi[2], M. Fujii[2], Y. Watanabe[2], K. Yagi[1]; [1]Osaka University, Osaka, Japan, [2]Showa Pharmaceutical University, Tokyo, Japan

Objective: Passing across epithelium is a first step for absorption of drug. There are tight junctions (TJs) between adjacent cells in the epithelium, overcome of TJs-barrier is essential for drug delivery. Claudin, a four-transmembrane protein, comprises a protein family consisting of more than 20 members and is a key component in TJs-barrier. Interestingly, expression and barrier-function of each member differ among tissues, suggesting that modulation of a claudin lead to deliver drug in a tissue-specific manner. In the present study, we investigated whether modulation of claudin is a promising method to deliver drugs using the C-terminal fragment of *Clostridium perfringens* enterotoxin (C-CPE), the only modulator of claudin-4. Methods: We prepared C-CPE and C-CPE mutants lacking their claudin-4 binding domain. To evaluate effects of C-CPE and C-CPE mutants on TJ-barrier and drug absorption, we measured trans-epithelial electric resistance in Caco-2 monolayer cell sheets, and we carried out in situ loop assay in rat jejunum using dextran as a model of drug. Dextran is absorbed through the paracellular route across epithelium. Results: Treatment of the cells with C-CPE disrupted TJs-barrier in time- and dose-dependent manner. C-CPE dose-dependently enhanced rat jejunal absorption of dextran. Notably, C-CPE was 400-fold more potent at the absorption-enhancing effect compared with sodium caprate, the only clinically used absorption-enhancer. Sodium caprate enhanced absorption in both jejunum and colon, whereas C-CPE did in jejunum but not in colon. C-CPE mutants lacking the binding region to claudin lost their activities to modulate claudin-barrier and enhance jejunal absorption. These results suggest that C-CPE modulated TJ-barriers and enhanced drug absorption through its interaction with claudin-4. Conclusion: Modulation of claudin is a novel promising method for drug delivery.

847/B44

Increment of Tight Junction Sealing by Epidermal Growth Factor is Regulated by Prostaglandin E2.

D. Flores-Benitez[1], R. Rincon-Heredia[2], L. F. Razgado[1], I. Larre[1], Y. De Lorenz[1], M. Cereijido[1], R. G. Contreras[1]; [1]Department of Physiology, Biophysics and Neurosciences, CINVESTAV, Mexico, [2]Department of Pharmacology, CINVESTAV, Mexico

Epithelia can adjust the permeability of their paracellular permeation route to physiological requirements, pathological conditions, and pharmacological challenges by, chiefly, regulating the degree of sealing of the tight junction (TJ). This is reflected by a transepithelial electrical resistance (TER) ranging from a few tenth to several thousands ohms per square centimeter, depending on the difference in composition between the fluid in the lumen and the interstitial fluid. The present work is part of a systematic search for the physiological causes of such regulation. We have previously shown that urine extracts from different animal species increment the TER of MDCK monolayers (Gallardo et al., 2002 J. Membrane Biol. 188:33), and that these effects are mediated by Epidermal Growth Factor (EGF) contained in urine (Flores-Benitez et al., 2007 Am. J. Physiol. 292:F828). The increase in TER is accompanied by an enhanced expression of claudin (cln)-4 and a decrement of cln-2. These changes are transitory, peaking at ~16 h, and returning to starting values at ~24 h. In this work we investigated how EGF provokes this transitory response. We found that (i) ERK1/2 activation by EGF is essential in both TER increase and cln-4 up-regulation, but (ii) does not appear to participate in cln-2 down-regulation. On the other hand, prostaglandin synthesis stimulated by EGF (iii) functions as a negative feedback, turning off the signal initiated by EGF. Thus, treatment of MDCK cells with prostaglandin E2 (PGE2) blocks ERK1/2 by a mechanism that involves αs heterotrimeric G protein, adenylyl cyclase and PKA.

848/B45

NHE-1 Inhibition by KR-33028 Attenuates Loss of BBB Integrity through Inhibition of Tight Junction Proteins Alteration.

S. Park[1,2], D. Lee[1,2], Y. Kim[1,2], M. Kim[1,2], S. Lee[4], K. Yi[4], S. Yoo[4], K. Lee[5], Y. Kim[5], S. Lee[1,3], E. Baik[1,3], C. Moon[1,3], Y. Jung[1,2]; [1]Physiology, Ajou University, Suwon, South Korea, [2]Brain Korea 21 for Molecular Science and Technology, Ajou University, Suwon, South Korea, [3]Brain Korea 21 for Medical Sciences, Ajou University, Suwon, South Korea, [4]Medical Science Division, Korea Research Institute of Technology, Ajou University, Daejon, South Korea, [5]Research Center, Yuyu Inc., Suwon, South Korea

Cerebral ischemia after stroke is responsible for the loss of blood-brain barrier (BBB) integrity resulting in an increase of vascular permeability and subsequently the formation of brain edema. As an essential structural component of the BBB, tight junctions (TJs) are located between brain endothelial cells, which consist of transmembrane and cytoplasmic proteins that are associated with the actin cytoskeleton. Na+/H+ exchanger (NHE)-1 is one of the most important pHi regulation systems in various cells. Recently, The NHE-1 inhibitors have been reported to protective effect against the ischemic brain edema. However, the precise mechanism remains unclear. In the present study, we investigated the effect of NHE-1 inhibition by KR-33028 on the BBB dysfunction, such as permeability increase and TJs alteration induced by ischemic insult in vivo and in vitro. In rat brain middle cerebral artery (MCA) occlusion model, NHE-1 inhibition significantly attenuated vascular leakage and loss of TJ proteins. In mouse brain endothelial cells (bEnd.3), not only the KR-33028 but also a Ca2+ chelator attenuated hypoxia-induced [Ca2+]i increase, BBB permeability increase and alteration of TJ proteins (occludin, ZO-1). And expression of TJs mRNA and total proteins was not changed during hypoxia in bEnd.3 cells. But alteration of TJ proteins is changed during

hypoxia. From these results, it is suggested that NHE-1 inhibition protects BBB integrity from permeability increase and TJ alteration through inhibition of [Ca2+]i overload during ischemia/hypoxia, and that the NHE-1 inhibition is likely to have preventive benefit of edema and therapeutic benefit in the treatment of ischemic stroke. This work was supported by a grant(CBM31-B3003-01-02-00) from the Center for Biological Modulators of the 21st Century Frontier R&D Program, Ministry of Education, Science and Technology, a grant (R-01-2005-000-10510-0) from Basic Research Program of the Korea Science and Engineering Foundation.

849/B46

AMPK Activation and GSK-3β Inhibition Independently Induce the Assembly of Epithelial Tight Junction Components in the Absence of Extracellular Calcium.

L. Zhang[1], J. Sfakianos[1], I. Mellman[1], M. Caplan[1,2]; [1]Department of Cell Biology, Yale University School of Medicine, New Haven, CT, [2]Department of Cellular and Molecular Physiology, Yale University School of Medicine, New Haven, CT

Recent studies have revealed that the tumor suppressor LKB1 is a kinase involved in the polarization of both epithelial cells and neurons. In a Drosophila model, the role of LKB1 in epithelial cell polarization is mediated by one of its substrates, AMP-activated protein kinase (AMPK), which is a sensor of cellular energy status. In a non-polarized neuron, LKB1 is selectively activated only in neurites destined to become axons, and this activation precedes the selective inhibition of GSK-3β at the same locations. GSK-3β, however, has not yet been identified to participate directly in epithelial cell polarization. Our previous studies found that the activation of AMPK initiates the relocalization of junction proteins to sites of cell-cell contact in the absence of extracellular calcium. In this study we found that in MDCK cells, the inhibition of GSK-3β by either SB216763 or LiCl is also capable of relocalizing junction proteins to sites of cell-cell contact independent of extracellular calcium. We also determined that the effects of AMPK activation and GSK-3β inhibition on the relocalization of junction proteins are synergistic and occur via different pathways. We found that knocking down expression of E-cadherin does not affect the calcium-independent relocalization of junction proteins induced by either AMPK activation or GSK-3β inhibition, and that knocking down expression of afadin disrupts the relocalization of junction proteins induced by AMPK activation, but not that induced by GSK-3β inhibition. We conclude that AMPK activation and GSK-3β inhibition independently induce the relocalization of epithelial junction proteins in the absence of extracellular calcium and that afadin appears to act downstream of AMPK to mediate this process.

850/B47

Hormone Ouabain Modulates Cell Adhesion: Effects on the Tight Junction.

M. I. Larre[1], O. Medina[2], C. Flores-Maldonado[1], R. Contreras[1], M. Balda[3], K. Matter[3], D. Flores-Benitez[1], M. Cereijido[1]; [1]Physiology, Biophysics and Neurocience, CINVESTAV, Mexico City, Mexico, [2]Biomedicine, CINVESTAV, Mexico City, Mexico, [3]Institute of Ophthalmology, University College Of London, London, United Kingdom

The high specificity of ouabain for Na+,K+-ATPase led to suspect that the organism might have an endogenous analogue. Recent work demonstrates that such substance exists, and is itself ouabain, a hormone whose physiological roles remain to be found. The purpose of our work is to find such roles. In the present work we concentrate on one of the types of cell-cell adhesion: the tight junction (TJ). We find that at 10 nM, a concentration within those usually found in mammalian tissues, ouabain does not inhibit the K+ pump of MDCK cells, but drastically modifies instead fundamental cell functions associated to cell adhesion: (1) it changes the levels of expression and pattern of cell distribution of components of the tight junction, such as claudins 1 and 2, while leaving undisturbed other molecular species of the TJ (i.e. occludin). (2) The effects on each claudin species occurs at a specific time of exposure to ouabain. (3) Changes in the molecular structure of the TJ are accompanied by a decrease of unidirectional flux of 3 Kd Dextran, but not of K+, and an increase of TER (transepithelial electrical resistance).

851/B48

Rhoa Mediates Altered Claudin-2 Expression in the Caco-2 Epithelial Model: a Role in Azaspiracid-1-Induced Barrier Disruption.

K. E. Cunningham, G. E. Ryan, T. McMorrow, J. Crean, M. P. Ryan; School of Biomolecular and Biomedical Science, UCD Conway Institute, Dublin, Ireland

Azaspiracids (AZAs) are a novel class of marine biotoxins found in shellfish that cause severe gastrointestinal (G.I.) disturbances in humans upon ingestion. Limited experiments in mice with chronic administration of Azaspiracid-1 (AZA-1) have been shown to cause G.I. epithelial damage. However the molecular mechanisms of AZA-1-induced alterations in the G.I. tract have not been elucidated. To explore potential mechanisms of AZA-1 toxicity in vitro, we utilised the Caco-2 intestinal epithelial model. When Caco-2 monolayers were exposed to AZA-1 (2.5-50nM), barrier function was significantly diminished between 4 and 72 hours, as measured by decreased transepithelial electrical resistance (TER) and increased apical to basolateral flux of fluorophore-conjugated dextrans (4-70 kDa). Cell viability was not reduced, suggesting a specific alteration to the tight junction complex. This study focused on the claudin family of tight junction proteins, as they constitute the major regulators of epithelial paracellular permeability. AZA-1 (10nM) increased claudin-2 and -4 mRNA and protein expression. Claudin-2, a pore-forming claudin highly expressed in leaky epithelia, was increased by AZA-1 in triton-X-100-insoluble protein fractions, suggesting increased cytoskeletal association. As AZA-1 has previously been shown to induce F-actin rearrangement in Caco-2 monolayers, and tight junctions are in part regulated by the cytoskeleton, we investigated a role for the Rho GTPases as mediators of AZA-1-induced barrier function loss. In a Rho pull-down assay AZA-1 caused an increase in active RhoA GTPase as early as 4 hours. Transfection with constitutively active (CA) RhoA(L63) caused an increase in claudin-2 expression. Expression of dominant negative (DN) RhoA(N19) reduced cytoskeletal-associated claudin-2 levels. RhoA(L63) expression also augmented the AZA-1 induced increase in claudin-2 in the triton-insoluble fraction. These data suggest RhoA mediates AZA-1-induced loss of barrier function in Caco-2 monolayers via increased claudin-2 expression, and provide new insights into the molecular mechanism of AZA-1 induced G.I. disturbances.

852/B49

DEP-1 Regulates Phosphorylation of Tight Junction Proteins and Enhances Barrier Function of Epithelial cells.

J. Sallee[1], K. Burridge[1,2]; [1]Cell and Developmental Biology, University of North Carolina Chapel Hill, Chapel Hill, NC, [2]Lineberger Cancer Center, Chapel Hill, NC

Cell-cell adhesion is a dynamic process that can activate several signaling pathways. These signaling pathways can be regulated through reversible tyrosine phosphorylation events. Levels of tyrosine phosphorylation of junctional proteins reflect the balance between protein tyrosine kinase and protein tyrosine phosphatase activity. The receptor tyrosine phosphatase DEP-1 (CD148/PTP-η) has been implicated in cell growth and differentiation as well as in regulating phosphorylation of junctional proteins. In this study, the catalytically dead substrate-trapping mutant of DEP-1 was used to identify potential substrates at cell-cell junctions. We have demonstrated that the trapping mutant of DEP-1 (DEP-1 D/A) interacts with the tight junction proteins occludin and ZO-1 in a tyrosine phosphorylation-dependent manner. In contrast, PTP-PEST, Shp2, and PTPμ did not interact with these proteins, suggesting that the interaction of DEP-1 with occludin and ZO-1 is specific. In addition, occludin and ZO-1 were dephosphorylated in vitro by DEP-1 but not by the other phosphatases tested. Over-expression of DEP-1 increased transepithelial electrical resistance in confluent epithelial monolayers and also reduced paracellular flux of FITC-dextran following a calcium switch. These data suggest that DEP-1 can modify the phosphorylation state of tight junction proteins and play a role in regulating permeability and junction formation.

853/B50

Arsenic Alters Tight Junction Function, Distribution and Expression in Human Airway Epithelial Cells.

C. L. Sherwood[1,3,4], A. E. Liguori[3,2], C. E. Olsen[3], R. Lantz[1,5,4], J. L. Burgess[6,5], S. Boitano[2,3,4]; [1]Cell Biology and Anatomy, Arizona Health Sciences Center, Tucson, AZ, [2]Physiology, Arizona Health Sciences Center, Tucson, AZ, [3]Arizona Respiratory Center, Arizona Health Sciences Center, Tucson, AZ, [4]Bio5 Research Institute, University of Arizona, Tucson, AZ, [5]Southwest Environmental Health Sciences Center, University of Arizona, Tucson, AZ, [6]Mel & Enid Zuckerman College of Public Health, Arizona Health Sciences Center, Tucson, AZ

The airway epithelium plays a role in the innate immune response, in part, due to a barrier that separates the air-filled lumen from the underlying tissue. Three categories of membrane spanning tight junction proteins contribute to the establishment of this barrier, including, the Junction Adhesion Molecules (JAMs), the Claudins, and Occludin. Arsenic is a natural metal toxicant that is prevalent in drinking water in the United States and throughout the world. Arsenic can accumulate in the lung after inhalation and/or ingestion. In animal and human studies, we and others have shown that arsenic ingestion can lead to altered lung function. These alterations include increased biomarkers in the lumen indicative of reduced wound repair and epithelial barrier dysfunction. In this report, we evaluated the effects of low-level acute arsenic exposure on airway epithelial barrier function, and specifically, on airway epithelial membrane spanning tight junction proteins. We found that low levels of arsenic in growth medium delayed (30-60 ppb) or prevented (290 ppb) the establishment of an airway epithelial monolayer *in vitro*. Addition of 290 ppb arsenic to pre-formed *in vitro* monolayers reduced transepithelial resistance within 24 hours, while exposure to 60 ppb arsenic for up to 4 days did not have a direct effect on transepithelial resistance. Using immunocytochemistry, immunoblotting and quantitative real time RT-PCR, we found that monolayers of airway epithelial cells exposed to 0, 60 or 290 ppb arsenic displayed altered and unique patterns of expression for JAM-A, several Claudins (1, 2, 3, 4, 5, 7), and Occludin. In summary, acute exposure to environmentally significant doses of arsenic can induce functional and structural changes in the airway epithelial barrier. These changes may be a compensatory reaction needed to maintain the integrity of the airway epithelial barrier and/or may lead to long-term effects on airway barrier properties and resulting physiology.

854/B51

Dynamic Regulation of Epithelial Adhesive Membrane Domain In Vivo.

K. Oshima, R. G. Fehon; Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL

The key function of epithelial cells is to isolate internal and external environments by forming a tight seal between cells. This function is provided by the tight junction (TJ) in vertebrate epithelia, while in invertebrates it is provided by the septate junction (SJ). Epithelial polarity is essential for formation of both structures, but the molecular mechanisms that establish these barriers are still largely unknown. In this study we examined the dynamic behavior of four GFP-tagged SJ components (ATPα, Nrv2, Nrg and NrxIV) *in vivo* using fluorescence recovery after photobleaching (FRAP). Our results indicate that the SJ is an exceptionally stable structure, showing recovery times much slower than the adherens junction component E-Cadherin. In contrast Dlg, a component of the basolateral polarity complex that colocalizes with the SJ, turned over more rapidly than SJ proteins, suggesting that it is not in the same protein complex as other SJ components. Interestingly, actin and microtubule inhibitors did not affect SJ stability, suggesting that cytoskeletal interactions are not important for SJ function. To further dissect the molecular interactions within the SJ, we analyzed the stability of GFP-tagged SJ proteins in the background of mutations in other SJ genes. As previously shown, GFP-SJ proteins mislocalize in *ATPα, nrv2, cor, nrxIV, nrg, mega, sinu, and gli* mutants, suggesting that all of the encoded proteins function in a single complex. Consistent with this idea, SJ proteins became unstable in the *nrv2, cor, nrxIV, nrg, mega and sinu* mutants. However, to our surprise we found that Nrv2 remains stable in the absence of ATPα, and Gli is dispensable for ATPα stability. Taken together these data suggest a model for SJ assembly in which a stable 'core SJ complex', including Nrv2, Cor, NrxIV, Nrg, Mega and Sinu, is formed away from the SJ, possibly in association with a secretory compartment, and then sorted to the correct membrane domain through the function of ATPα and Gli.

855/B52

HDACs Regulate Claudin-1 expression in Colon Cancer through Modulation of mRNA Stability.

M. Krishnan, A. B. Singh, P. Dhawan; Surgical Oncology, Vanderbilt University, Nashville, TN

Objective: Histone deacetylation and DNA methylation have a central role in the control of gene transcription resulting in repression of anti-tumor genes. It has been known that aberrant recruitment of HDAC activity is associated with the development of certain human cancers. Histone deacetylase inhibitors (HDACi) are promising anticancer agent that show efficacy against a variety of malignancies. Claudins, a large family of essential tight junction (TJ) proteins are abnormally regulated in human carcinomas, suggesting that they might represent potential targets for cancer detection, diagnosis and therapy. The objective of our study to examine possible role of HDAC-dependent epigenetic regulation of claudin-1 in colon cancer. Methods: We analyzed the effects of two HDAC inhibitors, Trichostatin A (TSA) and

Sodium Butarate(SB). The SW480 and SW620 cells treated with TSA and SB in dose dependent manner ((Untreated, Control, 50,100,200 and 500nM) for 36hours. RNA and protein samples were prepared and invasion assay performed by standard protocol. Result: HDACi inhibited Claudin-1 expression in multiple colon cancer cell lines in a dose and time-dependent manner at the RNA and protein levels. Genetic silencing of HDAC-2 and -3 expressions resulted in specific decreases in claudin-1 expression. Further studies revealed modulation of claudin-1 mRNA stability and not the mRNA transcription as the mechanism underlying HDAC-dependent claudin-1 expression in colon cancer cells. Most importantly, overexpression of Claudin-1 abrogated the TSA induced inhibition of invasion and apoptosis. In addition, our findings support a functional role of claudin-1 expression in TSA mediated regulation of invasion. Conclusions: Taken together, our studies demonstrate a novel mechanism of post transcriptional regulation of claudin-1 expression in colon cancer cells. Since HDAC inhibitors are used in epigenetic studies and are considered to be promising anticancer drugs, these new findings will have implications in both laboratory and clinical settings.

856/B53

The Effects of Long-Term Hyperoxia Exposure on Madin Darby Canine Kidney Cell Tight Junctions.

C. J. Hughbanks, R. A. Johansen, J. M. King; Biology, Trinity University, San Antonio, TX

Tight junctions located at the apicolateral space provide an important gate function limiting/regulating the transfer of ions and solutes through the paracellular space. Madin Darby Canine Kidney cells (MDCK-II) a model epithelium were oxidatively stressed using an oxygen chamber (ProOx111) calibrated to 95% O^2 and 5% CO^2. The hyperoxia treatment was administered to cultures of MDCK-II cells over long-term exposure (up to 72 hours). A variety of physiological parameters were measured to better understand junctional remodeling induced by oxidative stress. Cell proliferation was measured by determining DNA content, hyperoxia significantly depressed cell proliferation. Hyperoxia did not elevate apoptosis as determined by activated caspase-3 assays. Lactate Dehydrogenase (LDH) released from cells was significantly elevated at 72 hours of hyperoxia. This result indicates that plasma membrane integrity is compromised by hyperoxia results in MDCK-II cells. Tight junction integrity was evaluated by measuring transepithelial resistance (TER) and employing a fluorescein-flux assay. Significant decreases in TER and elevated paracellular flux suggest that hyperoxia conditions have detrimental effects on the tight junction. In order to determine specific effects on the tight junctions of MDCK-II cells stably expressing GFP-occludin and GFP-claudin-1 were employed to examine localization of these targets. RT-PCR studies are in progress to address changes in gene expression for tight junction targets and recovery enzymes such as heme oxygenase. Hyperoxia has modest effects on MDCK-II cells, this model may be useful for probing junctional remodeling due to oxidative stress. This work was supported by NIH Grant DK065652 and the TU- HHMI Grant.

857/B54

Tight Junction Protein Cleavage by Matrix Metalloproteinase-9 Results in Barrier Dysfunction.

J. C. Hickey, C. L. Koehler, J. M. King; Biology, Trinity University, San Antonio, TX

This study focuses on epithelial barrier function after exposure to proinflammatory cytokines (TNFα and IFNγ) or recombinant matrix metalloproteinase-9 (recMMP-9) in a model renal epithelial cell line (Madin-Darby Canine Kidney cells). We have shown that in response to proinflammatory cytokine challenge, MMP-9 secretion and activity is elevated in MDCK-II cells. Additionally, elevated secretion of MMP-9 into the apical compartment along with compromised barrier function was observed. The aim is to elucidate the mechanisms by which MMP-9 action leads to modification of the tight junction (TJ). In this study, limited proteolysis experiments were conducted using MDCK-II membrane preparations treated with recMMP-9, Western Blot analysis demonstrated that claudin-1 was cleaved. Substrate analysis revealed a putative MMP-9 cleavage site within the first extracellular loop of claudin-1. Stably expressing GFP-claudin-1 and GFP-occludin MDCK-II cell lines were generated and TJ barrier properties including transepithelial resistance and paracellular flux were analyzed in these cell lines. These lines were then used to analyze effect of recMMP-9 exposure on claudin-1 and occludin localization. Site-directed mutagenesis was employed to modify the putative MMP-9 claudin-1 cleavage site. Three additional GFP-claudin-1 mutant cell lines were generated containing a potentially null site or sites more closely resembling the consensus MMP-9 site. The effect of recMMP-9 exposure on claudin-1 localization was examined by fluorescent microscopy using these lines. These studies indicated increased fragmentation of claudin-1 by recMMP-9 with minimal changes to occludin localization. This work was supported by NIH Grant DK065652 and the TU- HHMI Grant.

Organization & Regulation of the Extracellular Matrix (858 – 871)

858/B55

Polyelectrolyte-Coated Gold Nanorods Modulate Collagen Fibrillogenesis and Mechanical Properties.

C. Wilson[1], P. N. Sisco[2], J. Hemmer[3], D. M. Dean[3], C. J. Murphy[2], E. Goldsmith[1]; [1]Cell Biology and Anatomy, University of South Carolina School of Medicine, Columbia, SC, [2]Chemistry and Biochemistry, University of South Carolina, Columbia, SC, [3]Bioengineering, Clemson University, Clemson, SC

Nanoparticles have demonstrated utility in imaging, targeting, and drug delivery, and novel surface chemistries have reduced the cytotoxic effects of these materials. At the same time, it has become clear that cells are sensitive to nanoscale variations in substrate topography and adhesion ligand clustering. In addition, several matrix proteins exhibit self-assembly under physiologic conditions to generate networks with characteristic dimensions on the nanoscale. However, the capacity for nanoparticles to elicit meaningful biophysical responses, such as modulation of matrix protein organization, has not been determined. The objective of this work was to investigate the effects of gold nanorods on type I collagen polymerization and mechanical properties. Gold nanorods (392nm long x 22nm wide) were prepared via seed-mediated, surfactant-directed synthesis. The nanorods were coated with alternating layers of the polyanion poly(styrene sulfonate) (PSS) and the polycation poly(diallyldimethylammonium chloride) (PDADMAC) to yield PSS- or PDADMAC-coated nanorods. Various concentrations of polyelectrolyte-coated nanorods were added to ice cold neutralized type I collagen, and polymerization at 37C was monitored by measuring the absorbance at 400nm. Subsets of gels were fixed and examined by confocal and electron microscopy. The mechanical properties of the unfixed gels were determined by atomic force microscopy. The highest concentrations of PSS-coated nanorods

attenuated the lag phase of collagen fibrillogenesis by 50%, whereas low concentrations of PDADMAC-coated nanorods yielded peak delays in the lag phase by 16%. Reflectance microscopy revealed a distinct tendency for PDADMAC-coated rods to aggregate in the polymerized collagen, although collagen network morphology was similar between groups. Electron microscopy indicated that the morphology of collagen fibers was also similar between nanorod-doped and control gels, although particles were found on the surfaces of some fibers. The apparent stiffness of gels containing PSS-coated nanorods was 64% lower than control gels. These results suggest that polyelectrolyte-coated nanomaterials can perturb collagen self-assembly and thereby influence cell-matrix interactions.

859/B56

Strategy to Design a Stratified Lamellar Corneal Construct.

J. Shankardas[1,2], R. Arafeh[1], A. A. Sule[1,3], S. Dimitrijevich[1]; [1]Integrative Physiology, UNTHSC, Fort Worth, TX, [2]Molecular Biology, UNTHSC, Fort Worth, TX, [3]Graduate School of Biomedical Sciences, UNTHSC, Fort Worth, TX

Purpose: We hypothesize that lamellar organization of collagen in the corneal stroma may be reproduced in vitro and that it determines optical clarity and cellular organization. By incorporating poly-methylmethacrylate (PMMA) membranes into the construct, we will determine the feasibility of assembling a biocompatible corneal prosthesis with appropriate physical properties. Methods: Neutralized collagen type I solution and PMMA coated glass coverslips produced thin films. Each film was seeded with normal human keratocytes (by 1x105 cell/film) which had been labeled with Cell Tracker Green® and Cell Tracker Orange® for 24 hours. Collagen films were then peeled off the coverslips and assembled as alternating CTG and OTG layers into lamellar stacks to mimic the stroma's structure. Scanning laser confocal microscopy was used to show the alternating layers of red and green fluorescently labeled cells and the cell alignment. Expression of cytoskeletal proteins such as: smooth muscle actin, myosin heavy chain, myosin light chain and vimentin in keratocytes on collagen and PMMA were compared with human corneal sections. Results: A stack of 20 collagen films was achieved using the above protocol. Scanning laser confocal microscopy showed the series of lamellae carrying cells. The keratocytes on collagen/PMMA have a similar cytoskeletal expression profile to those in the human cornea and were aligned in orthogonal arrangement from one layer to the next. The light transmittance and polarization of the light passing through the constructs were determined. Conclusions: It is possible to efficiently and reproducibly assemble lamellar stroma model composed of up to 20 layers of collagen (~ 150 µm thick) and PMMA populated by keratocytes. The cells cultured on the collagen films were shown to adopt a parallel unidirectional alignment in accordance with the proposed alignment of collagen fibrils. The individual films are assembled so that the orthogonal lamellar arrangement of collagen fibers and cells characteristic of the corneal stroma in vivo is reproduced. Immunohistochemsitry staining showed that the protein expression of the keratocytes on the collagen and PMMA construct is similar to that in vivo.

860/B57

The Extracellular Matrix as a Charge Barrier - An Electrostatic Bandpass Filter.

O. Lieleg, R. Baumgärtel, A. R. Bausch; Physics Department, TU München, Lehrstuhl E27 für Zellbiophysik, Garching, Germany

Cells produce extracellular biopolymers such as collagens and proteoglycans to create a scaffold that promotes adhesion and proliferation. Besides its structural tasks the extracellular matrix (ECM) is supposed to shape and regulate the distribution of many diffusing ions, proteins and drugs and to provide means for the controlled storage and release of growth factors. However, the molecular basis for these regulation mechanisms is poorly understood. Here, we demonstrate in an in vitro study that the ECM is an electrostatic bandpass filter. We report a drastic reduction of the diffusion coefficient of both negatively and positively charged particles in ECM by three orders of magnitude to virtually zero mobility. In contrast, uncharged particles can diffuse almost freely within the ECM. The observed effect is independent from the size of the test particle but depends sensitively on its surface charge density. Consistently, the electrostatic filtering can be suppressed and the mobility of the particles is rescued at high ionic strengths, i.e. if the surface charge of the diffusing particle is screened. A similar remobilization of trapped particles can be induced by heparin, however only for positively charged particles. Our results indicate that distributions of localized charge patches may be responsible for the highly selective but unspecific filtering effect of the ECM.

861/B58

Aberrant Methylation-Associated Downregulation of 3-O-Sulfotransferase (3-OST) Genes Alters Heparan-Sulfate Proteoglycans Structure and Function in a Human Chondrosarcoma Cell Line.

C. Bui[1,2], M. W. Coughtrie[2], S. Sharp[2], P. Netter[1], J. Magdalou[1], M. Ouzzine[1], S. Fournel-Gigleux[1,2]; [1]UMR 7561-UHP Nancy1, University of Nancy, Vandoeuvre les Nancy, France, [2]Pathology and Neuroscience, University of Dundee, Dundee, United Kingdom

Located at the cell-tissue-organ interface, heparan-sulfate proteoglycans (HSPGs) facilitate ligand-receptor interactions crucial to many physiological processes. They are as well involved in the pathogenesis of various diseases. There is growing evidence that HSPGs are implicated in several aspects of cancer biology, including cell transformation, tumour invasion and metastasis. The synthesis of glycosaminoglycan chains involves the coordinated action of an array of glycosyltransferase and sulfotransferase enzymes(1). HS-O-sulfotransferases catalyze the sulfation of saccharide residues at specific positions of HS chains, governing unique biological functions. Although the biosynthetic scheme of HSPGs has been outlined, little is known about the regulation of this complex process. Interestingly, hypermethylation of a CpG island in the 3-OST2 promoter in several primary tumours has been recently reported(2). The objective of this study is to determine whether 3-OST genes are subject to epigenetic modification via methylation and to analyze potential functional consequences in a chondrosarcoma cell line (HEMC). We determined the DNA methylation profile of 3-OST gene family by bisulfite conversion providing evidence for hypermethylation of 3-OST1, 3-OST2, 3-OST3A and 3-OST6 genes, while no CG methylated site was present in 3-OST3B promoter. Semi-quantitative RT-PCR analysis revealed that 3-OST mRNA expression levels were increased upon 5-aza-2'-deoxycytidine (5-Aza) treatment of HEMC cells indicating that aberrant methylation affects transcription of these genes. In order to evaluate the potential impact of an epigenetic regulation on HSPG synthesis, we analyzed cellular HSPGs content by DEAE-anion exchange and gel filtration chromatography. Our results show a modification of HSPGs sulfation pattern upon 5-Aza-dc treatment confirmed by immunofluorescence analysis. Our data suggest that aberrant methylation of 3-OST genes leads to altered HSPG structure in HEMC cells and

abnormal migration properties that may contribute to chondrosarcoma pathogenesis. (1) Gulberti et al J Biol Chem 2005, 280, 1417 (2) Miyamoto et al Oncogene 2003, 22, 274 Supported by INSERM-University of Dundee (C2I)/ANR GAGNetworkPCV08_321510

862/B59

Analysis of Three Regions of the Deep Digital Flexor Tendon of Rats Submitted to Immobilization and Immobilization Followed of Stretching.

E. R. Pimentel[1], A. A. Aro[1], B. C. Vidal[1], A. Biancalana[1], L. Gomes[1], S. M. Matiello-Rosa[2]; [1]Department of Cell Biology, UNICAMP, Campinas, Brazil, [2]Department of Physiotherapy, UFSCar, Sao Carlos, Brazil

Few studies have discussed the effect of immobilization and stretching on the structural and biochemical properties of tendons, which withstand tension and compressive forces. The purpose of this study was to analyze the effect of immobilization and stretching after a long period of immobilization on the proximal (p), intermediary (i) and distal (d) regions of the deep digital flexor tendon (DDFT) of rats. The p and d regions are under tension and the i region bears compression. Rats Wistar were separated into five groups: GI- control of immobilized rats; GII- immobilized; GIII- control of immobilized rats followed by stretching or allowing free cage activity; GIV- immobilized and stretched; and GV- immobilized and allowed free cage activity. The analysis of sections stained with toluidine blue showed very low metachromasy in the i region of GII, compared to GI, suggesting that immobilization results in diminishing glycosaminoglycans (GAG) presence in the compression region. In the same region in GIV and GV, a strong metachromasy was observed, besides the presence of chondrocyte like cells. The concentration (µg/mL) of dermatan sulfate (DS) in the i region of GII, determined by densitometry in agarose gel, was smaller (7.09±0.46) in relation to the control GI (9.64±0.02); no chondroitin sulfate (CS) was detected. In the same region, in tendon of GIV, the concentrations of CS and DS were respectively 7.57±0.03 and 10.53±0.02, whereas in the control GIII, we found 3.14±0.2 and 7.71±0.3 respectively. With respect to the amount of non collagenous proteins, a significant decrease was observed in the 3 regions of the DDFT submitted to immobilization; however with exception to the p region, higher values than the control were observed when the tendon was submitted to stretching after immobilization. Result of SDS-PAGE did not show remarkable differences among the groups. Our results suggest that both stretching and free activity are efficient in the recovery of GAGs and non collagenous proteins in the DDFT of rats previously immobilized.

863/B60

A Structural and Biochemical Study on the Effect of Immobilization followed by Stretching of the Achilles Tendon of Rats.

A. A. Aro[1], B. C. Vidal[1], T. C. Tomiosso[1], S. M. Matiello-Rosa[2], L. Gomes[1], E. R. Pimentel[1]; [1]Department of Cell Biology, UNICAMP, Campinas, Brazil, [2]Department of Physiotherapy, UFSCar, Sao Carlos, Brazil

There is little information concerning the effect of stretching on the biochemical and morphological features of tendons submitted to a long period of immobilization. Our purpose was to evaluate the response of rat tendons to stretching procedures after immobilization. The experimental groups were: GI- control of immobilized rats; GII- immobilized; GIII- control of immobilized rats followed by stretching or allowing free cage activity; GIV- immobilized and stretched rats; GV- immobilized and allowed free cage activity. Analysis in SDS-PAGE showed no remarkable differences among the groups, but it is worthwhile to note that the 35, 32, 18, and 17 kDa proteins were more prominent in compression regions than in tension regions, regardless of the groups. Analysis of hydroxyproline, showed a larger concentration (mg/g tissue) in the compression region of GII (50±3), in relation to the control GI (38±2), whereas in GIV and GV had less hydroxyproline (32±4) than the control GIII (45±2). With respect to glycosaminoglycans (GAG), a remarkable increase was found in the compression region of GIV (4.2±0.2) in relation to the control GIII (2.7±0.4), especially in the quantity of chondroitin sulfate. The presence of dermatan sulfate was outstanding in the compression and tension regions of the GII and GV groups. In regard to the concentration of non-collagenous proteins, differences were detected only in the tension region, where a larger concentration was found in GII (47±5 mg/g tissue). In the ponceau SS stained sections, analyzed under polarization microscopy, GII exhibited the highest disorganization of collagen bundles, which is partially recovered after stretching or only with remobilization, as confirmed after Linear Dichroism (LD) measurements. Analysis of LD of sections stained with toluidine blue, showed that immobilization diminished the alignment of the GAG in relation to the collagen bundles, however this situation was reverted when remobilization was allowed. Our results suggest a revision should be undertaken in the stretching procedures, in terms of duration and periodicity of the sessions.

864/B61

Transforming Growth Factor β Induces Extracellular Matrix Protein Crosslinking Enzyme Lysyl Oxidase and Other LOX Family Proteins.

A. Sethi[1], M. Waggoner[2], A. Taylor[1], R. J. Wordinger[1], A. F. Clark[1]; [1]Cell Biology & Genetics, U. North Texas Health Science Center, Ft. Worth, TX, [2]Texas College of Osteopathic Medicine, UNTHSC, Ft. Worth, TX

OBJECTIVE: Glaucoma is a leading cause of irreversible blindness in the world and is associated with elevated intraocular pressure (IOP) due to increased aqueous outflow resistance in the trabecular meshwork (TM). Increased deposition of extracellular matrix (ECM) material in the TM appears to be responsible for this glaucomatous IOP elevation. Lysyl oxidase (LOX) is an extracellular matrix crosslinking enzyme, and recent genome wide association studies showed that SNPs in LOXL1, a LOX family member, significantly increased the risk of developing exfoliation glaucoma. Transforming growth factor beta (TGFβ) is elevated in glaucoma patients, and can cause increased ECM deposition in the TM and elevated IOP. Our lab has recently showed that TGFβ-2 induced tissue transglutaminase (TGM2), another family of cross linking enzymes, in humanTM cells. The objective of the current study is to evaluate the effects of exogenous TGFβ (1, 2 and 3) treatment on LOX and LOXL1-4 mRNA and protein expression in cultured human TM cells. METHODS: Primary glaucomatous and normal TM cells were grown in DMEM containing 10% FBS until 80% confluent and transferred to serum free medium for 24hrs prior to TGFβ treatment. Both cell associated and secreted LOX protein levels were analyzed using western immunoblotting. mRNA levels were determined by quantitative-RT-PCR. RESULTS: After 48hrs, protein levels of both LOX and LOXL2 were induced by all three isoforms of TGFβ (5ng/ml). TGFβ-2 elevated LOX and LOXL2 levels in the conditioned medium. TGFβ-2 also caused a dose dependent increase in both secreted and cell associated LOX and LOXL2 proteins. We are examining the expression of other members of the LOX family (LOXL1,

LOXL3, LOXL4) using q-RT-PCR. CONCLUSION: Our studies indicate that there is an increased induction of LOX and LOXL2 proteins in TM cells treated with TGFβ and that TGFβ-2 can cause secretion of these proteins in a dose dependent fashion.

865/B62

Loss of BRM Expression Contributes to a Tumor-Like Phenotype via Enhanced A5β1 Integrin Expression and Activity.

K. M. Stewart[1], N. Cohet[2], D. Reisman[3], J. Lakins[1], G. I. Rozenberg[4], A. N. Imbalzano[2], J. A. Nickerson[2], V. M. Weaver[1]; [1]Surgery, University of San Francisco, San Francisco, CA, [2]Cell Biology, University of Massachusetts Medical School, Worcester, MA, [3]Internal Medicine, University of Michigan, Ann Arbor, MI, [4]Genetics, University of North Carolina School of Medicine, Chapel Hill, NC

α5 integrin and its receptors such as the fibronectin receptor are critical for normal mammary epithelial cell (MEC)-extracellular matrix (ECM) interactions and are commonly perturbed in human breast tumors. In addition, elevated fibronectin levels are associated with breast tumors and the metastatic process. The mechanisms governing α5β1 expression, however, remain poorly defined. Recent evidence has demonstrated that loss of function of one of the catalytic subunits of the SWI/SNF chromatin remodeling complex, BRG-1, promotes an aggressive metastatic behavior in human fibroblasts via increased expression of α5 and αV integrins. Expressions of BRG-1 and the other SWI/SNF catalytic subunit, BRM, are often reduced or completely lost in tumors, including that of the lung, prostate and breast. Microarray data mining revealed that the mRNA levels of BRM are inversely related to breast tumor grade. We investigated the functional consequences of shRNA-mediated knockdown of BRM on non-transformed MEC behavior through the application of two- and three-dimensional reconstituted basement membrane (2D and 3D rBM) culture assays. Data showed that loss of BRM expression enhanced adhesion and migratory behavior of MECs grown on 2D extracellular substrata. Additionally, loss of BRM expression perturbed normal tissue morphogenesis of MECs grown within 3D rBM, reflected by a significant increase in colony size, loss of lumen formation, and enhanced secretion and deposition of fibronectin. These data suggest that BRM may play a critical role in modulating MEC-ECM interactions to alter cell behavior in the pathogenesis of breast cancer via regulation of integrin expression. (Support: DODWX81XWH-05-1-0330)

866/B63

Syndecan-1 Ectodomain Shedding is Regulated by the Small GTPase Rab5.

K. Hayashida, P. Park; Division of Respiratory Diseases, Children's Hospital Boston, Harvard Medical School, Boston, MA

A major cell surface heparan sulfate proteoglycan, syndecan-1 can be proteolytically cleaved at extracellular domain and release functional ectodomain (shedding) to regulate molecular and cellular processes central to the pathogenesis of inflammatory diseases. Several extracellular agonists have been identified to induce syndecan-1 shedding and metalloproteinases have been shown responsible for the proteolytic cleavage. This shedding process is highly regulated where outside-in signaling and intracellular signaling molecules are suggested to be involved. However, the intracellular mechanisms in detail that regulate syndecan-1 shedding are largely unknown. Here we examined the role of syndecan-1 cytoplasmic domain in the regulation of agonist-induced syndecan-1 shedding. Our results indicated that the syndecan-1 cytoplasmic domain was essential because mutation of invariant Tyr residues abrogates ectodomain shedding, but not because it is Tyr phosphorylated upon shedding stimulation. Instead, Co-Immunoprecipitation by anti-syndecan-1 monoclonal antibody and co-sedimentation assay with recombinant syndecan-1 cytoplasmic domain showed that the syndecan-1 cytoplasmic domain binds to Rab5, a small GTPase that regulates intracellular trafficking and signaling events. GST pull down assay showed Syndecan-1 cytoplasmic domain bound specifically to Rab5 and preferentially to inactive GDP-Rab5a over active GTP-Rab5a. Shedding stimulation induced the dissociation of Rab5 from the syndecan-1 cytoplasmic domain. Moreover, the expression of dominant-negative Rab5a interfered with the stimulation-induced dissociation of Rab5 from the syndecan-1 cytoplasmic domain and significantly inhibited syndecan-1 ectodomain shedding induced by several distinct agonists. Based on these data, we propose that Rab5 is a critical regulator of syndecan-1 shedding that serves as an on-off molecular switch through its alternation between the GDP-bound and GTP-bound forms.

867/B64

IsoDGR Sequences do not Mediate Binding of Fibronectin N-terminus to Adherent Fibronectin-null Fibroblasts or Platelets.

J. Xu, L. Maurer, B. Hoffmann, D. S. Annis, D. Mosher; Biomolecular Chemistry, University of Wisconsin-Madison, Madison, WI

Fibronectin (FN) is assembled by mouse embryos even when the RGD sequence of 10F3 module is replaced by RGE (FN-RGE), causing loss of the principal α5β1 integrin-binding site (Takahashi et al., 2007a). Spontaneous conversion of N263GR in module 1F5 and N501GR in 1F7 sequences in the 1F5 and 1F7 modules to isoDGR recognized by αvβ3 integrins possibly explains this enigma, i.e., ligation of isoDGR by αvβ3 allows cell to concentrate and manipulate assembling FN(Takahashi et al., 2007a). To assess this hypothesis, we first assayed for the presence of isoDGR in purified plasma fibronectin. Modification of N263GR sequence was found. We then generated mutations of NGR sequences in FN, FN-RGE, and the N-terminal 70K FN fragment including 1F5 and 1F7 (70K FN) and studied their assembly and binding abilities. FN-N263/501Q with both NGRs mutated to QGRs, was assembled by either platelets or fibronectin-null fibroblasts. FN-RGE and FN-RGE-N263/501Q were assembled by platelets or fibronectin-null fibroblasts, although to a lesser extent than FN and FN-N263/501Q. These results indicate that NGR sequences are not required for FN or FN-RGE assembly. Mutations of either one or both of the NGR sequences of 70K FN did not affect the binding of 70K FN to platelets or fibronectin-null fibroblasts. We conclude that the isoDGR sequences spontaneously converted from NGR sequences do not mediate the interaction between 70K FN and the cell surface and are not required by FN assembly in the absence of an intact RGD sequence.

868/B65

Preparation of HA-Probes for Imaging Aggressive Tumor Cells.

M. Veiseh[1], Y. Qi[2], J. Zhang[2], M. Bissell[1], E. A. Turley[2]; [1]Life Sciences, Lawrence Berkeley National Laboratories, Berkeley, CA, [2]Oncology, University of Western Ontario, London, ON, Canada

Increased hyaluronan (HA) production and degradation is often a feature of epithelial and fibroblast tumors. Furthermore increased HA in both the tumor epithelium and peri-tumor stroma of breast malignancies is independently associated with poor clinical outcome. We reasoned that this modified HA accumulation likely reflects altered uptake and metabolism, which can be detected by monitoring uptake of an

exogenous, labeled-HA probe. Our objectives were to prepare HA-based fluorescent imaging probes, determine if these are taken up to a greater extent by transformed fibroblasts and tumor epithelium than their less tumorigenic counterparts and identify the HA receptors that mediate uptake of the probe. Transformed fibroblasts lines (e.g. mutant active Ras-10T1/2; RHAMM-10T1/2), parental lines (10T1/2) and a panel of human breast cancer epithelial lines that differed in their phenotypes (e.g. basal or luminal) and aggressiveness were analyzed. Probe uptake was highest in Ras or RHAMM-transformed cells compared to non-tumorigenic fibroblasts and was a receptor-mediated process because it was competed by exogenous unlabeled HA and required a minimum of 12-30 saccharides. It was also mediated by endocytosis since it was blocked by inhibitors of this process. Uptake rates in transformed fibroblasts did not correlate with display of CD44 but rather with RHAMM. Nevertheless, the use of blocking antibodies and genetic deletion of CD44 established that blocking/ablating CD44 function completely inhibited HA probe uptake while blocking/ablating RHAMM function/expression reduced probe uptake to levels of untransformed fibroblasts. These results suggest that HA-probe uptake in fibroblasts uses two transport mechanisms: CD44 is essential for the uptake to occur, but the increased uptake is mediated by RHAMM plus CD44. High uptake of HA probe was also observed in sub-populations of aggressive breast cancer cell lines such as MDA-MB-231 and ongoing studies are designed to identify the HA receptors involved in this high uptake and to determine the nature of the subpopulation. We propose that increased uptake of HA probe may be a useful tool for both identifying/imaging and targeting aggressive tumor cell subsets.

869/B66

Functional Haplotype of Collagen Type III Alpha 1 in a Korean Population Was Associated with Susceptibility to Chronic Hepatitis B and Cirrhosis.

S. Lee[1], C. Yi[1], M. Kim[1], J. Cheong[2], S. Cho[2], S. Yang[3], H. Kim[1], K. Kim[1], J. Kim[1], A. Oh[1], J. Pyun[1], K. Lee[1], H. Song[1], S. Won[1], K. Kwack[1]; [1]Medical Genomics Laboratory, CHA Research Institute, Pochon CHA University, Seongnam, South Korea, [2]Department of Gastroenterology, Genomic Research Center for Gastroenterology, Ajou University School of Medicine, Suwon, South Korea, [3]Macrogen Inc, Seoul, South Korea

Objective: Hepatitis B virus infection has been recognized as one of the major risk factors for liver diseases including cirrhosis and hepatocellular carcinoma. It is a major public health problem worldwide owing to life-threatening complications of portal hypertension and liver failure and appears to increase the risk of incident hepatocellular carcinoma. In healthy liver, the homeostasis of extracellular matrix is achieved via the dynamic balance between the synthesis (fibrogenesis) and breakdown (fibrolysis) of ECM. However, excessive accumulation of ECM can occur due to prolonged liver injury by HBV infection. Collagen type III alpha 1 (COL3A1) is one of the extracellular matrix proteins. The expression of COL3A1 is closely related to viral and non viral liver diseases. Methods: Here, we investigated whether single nucleotide polymorphisms (SNPs) of COL3A1 confer genetic susceptibility to patients with chronic liver diseases. A total of 399 Korean people, 111 patients with chronic hepatitis B (CH), 95 patients with liver cirrhosis (CIR), 86 patients with hepatocellular carcinoma (HCC), and 107 spontaneously recovery (SR), were genotyped for 16 SNPs of the COL3A1 gene. Results: The A allele of rs3106796 was highly associated with the CH (p=0.01), CIR (p=0.01), and HCC (p=0.03). Haplotype 3 of the LD block 2 was significantly associated with CH (p=0.02), CIR (p=0.03), and HCC (p=0.03). Diplotype analysis showed that they had increased relative risk for CH and CIR in the two diplotypes, dt3 (p=0.01) and dt6 (p=0.01; and p=0.05) against dt1, the most common diplotype in both Korean groups. In vitro reporter gene assays showed that the constructs containing the G allele of rs3106796 appear to exert lower transcriptional activity of COL3A1 than the A allele, depending on the promoter types. Conclusions: Functional SNP and haplotypes of COL3A1 may be genetic factors that increase the susceptibility of Koreans to chronic HBV infection and cirrhosis.

870/B67

Chondroitin Proteoglycans are Required for C. elegans Eggshell Assembly.

S. K. Olson[1], T. Muller-Reichert[2], A. B. Desai[1], K. Oegema[1]; [1]Ludwig Institute for Cancer Research, University of California, San Diego, La Jolla, CA, [2]Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

The nematode eggshell is a rigid, impermeable structure that protects the embryo during development. The essential function of the eggshell makes it an attractive target for the development of drugs to combat parasitic nematode infection, which impacts the health of over 2 billion people. However, this approach is limited by what little is known about the composition of the nematode eggshell or the process by which it forms. We are using *C. elegans* as a model system to study nematode eggshell assembly, and have identified two genes that are required redundantly for eggshell formation. *cpg-1/cej-1* and *cpg-2* encode chondroitin proteoglycans, a class of secreted extracellular matrix glycoproteins post-translationally modified with chondroitin sugar chains. CPG-1 and CPG-2 localize to the eggshell in immunofluorescence and immuno-electron microscopy experiments, which suggests a structural role for these proteins in the eggshell. Our data point to a distinct role for the CPG core proteins in eggshell assembly that is separable from the role of the associated chondroitin chains. Ultra-structural analysis shows that embryos co-depleted of CPG-1 and CPG-2 form fewer eggshell layers than chondroitin-depleted embryos (which still express naked CPG core proteins). Additionally, a fluorescence microscopy-based assay was utilized to investigate the effect of eggshell defects on early embryonic development. Embryos depleted of either CPG-1/CPG-2 or chondroitin have identical phenotypes *in utero*, exhibiting defects in osmotic integrity and cytokinesis. However, osmotic support rescues early embryonic events (including cytokinesis) robustly in chondroitin depletions, but only partially in CPG-1/CPG-2 co-depletions. The correlation of structural and functional data suggests that the CPG core proteins have a distinct role in eggshell assembly that is separable and upstream of the role of the associated chondroitin chains.

871/B68

Characterization of a Tissue-engineered Human Extracellular Matrix for Cell Culture Applications.

F. Zeigler, K. Nickey, J. Harris, G. Naughton; histogen, Inc., Encinitas, CA

Primary human dermal fibroblasts in scalable fermentation-style bioreactors grown in defined, animal component-free medium, have been used in the production of human basement membrane extracellular matrix (ECM). Some of the key components of a fetal-like, undifferentiated mesenchymal tissue have been produced. ECM proteins found in basement membranes with a known role in tissue

development and regeneration are also produced, including laminin and collagen type IV, along with fibronectin, tenascin, and collagen type V, a fibrillar collagen only found at high abundance during fetal development. Utilizing hypoxia to upregulate embryonic ECM protein production during tissue formation in vitro in stirred bioreactors, a very early embryonic environment has been recapitulated. This hypoxic culture system also enhances the secretion of a wide variety of regeneration-associated growth factors and key developmental regulators of stem cell pluripotency and differentiation, such as the wnt gene family. This novel technology offers a commercially-viable source of human ECM for a wide variety of in vitro cell culture applications including pluripotent human embryonic stem cell culture, where we show maintenance of pluripotent phenotype by FACS analysis of Oct-4 expression in BGO1v cells. We also show that these human BMZ proteins maintain primary human hepatocyte phenotype, and also support the expansion in culture of variety of commonly used cell types which generally rely on murine tumor BMZ extracts such as Matrigel to support phenotypic human cell cultures for cell biology studies.

Extracellular Matrix & Cell Behavior I (872 – 891)

872/B69

Biocompatibility of the New Biomaterial Derived from Porcine Small Intestine Submucosa.

F. de Paoli[1, 2], M. Gatti[2], B. Vidal[2]; [1]Centro Universitario Serra dos Orgaos, Rio de Janeiro, Brazil, [2]Universidade Estadual de Campinas, Campinas, Brazil

The purpose of this study was to evaluate the biocompatibility of the membrane derived from porcine small intestine submucosa (SIS), characterizing the potential of this device composed by extracellular matrix (EM) as a biomaterial.Born hamster kidney cells (BHK-21)were cultured on SIS-membrane pieces with 90% D-MEM medium, supplemented with 10% fetal bovine serum, 10.000UI/ml penicillin and 100 mg/ml streptomycin.The samples were incubated at 37°C and 5% CO2 for 72 h.Cells cultured in absence of SIS-membrane were used as controls.SIS-membrane with cells culture and control cells were fixed in ethanol/acetic acid solution, submitted to Feulgen-reaction and studied by image analysis. The geometric (area) and densitometric (optical density OD, standard deviation of gray average SDtd and integrate optical density IOD) parameters were calculated and submitted to Mann-Whitney test.BHK-21 cells were observed preferentially on collagen fibers. The image analysis demonstrated that BHK-21 responded to SIS-membrane through of the nuclei-phenotypic modification. The value of the area decreased in cell cultured on SIS-membrane, while IOD, OD and SDtd values were increased.Higher values of IOD may be correlated with the cellular differentiation, hypothesizing that those cells can be able to synthesize new components. The area-IOD relation of the BHK-21 cells cultured on SIS-membrane demonstrated two distinct populations of cells, indicating the tendency to BHK-21 cells-like fibroblasts (small area and heterogenic chromatin). Chromatin heterogeneity was measured by OD and SDtd values that reflect to euchromatin condensation.Higher OD values were found in cells cultured on the SIS-membrane and indicated a high order of chromatin organization. This condensation facilitates the proximity of active chromatin domains and may help these cells to respond to stimuli through factors remains. The present study demonstrated that BHK-21 cells cultured on the SIS-membrane presented a good biocompatibility due a good response of the adherence, migration and differentiation.This study also suggests the used of the SIS-membrane as biomaterial.

873/B70

Dock5 an Essential Rac Exchange Factor in Osteoclasts that Controls Adhesion Structures Organization and Bone Resorbing Activity.

A. Blangy[1], H. Brazier[1], G. Pawlak[1], V. Vives[1], M. Laurin[2], J. Cote[2]; [1]CRBM UMR 537, CNRS, Montpellier Cedex 5, France, [2]Institut de Recherches Cliniques de Montréal, Montreal, Montreal, QC, Canada

RhoGTPase signaling pathways have essential functions in osteoclast biology. Dock5 belongs to the Dock/CZH family of exchange factors, a group of proteins that activate RhoGTPases. It is the closest homolog to Dock180. We reported previously that the expression of Dock5 increased during RANKL-induced osteoclast differentiation and that its suppression was not compatible with cell survival during osteoclastogenesis (Brazier et al, JBMR, 2006, 21(9), 1387). We now show that Dock5 mRNA is predominantly expressed in osteoclasts and to a lesser extend in placenta. Its level of expression is very low in all other mouse tissues tested including mammary gland and total bone marrow. By GTP-trapping assays, we also found that Dock5 is an activator of Rac. As its closest homolog Dock180, Dock5 guanine nucleotide exchange activity is inhibited by its amino-terminal SH3 domain and it can be activated through its binding to the adaptor protein Elmo. In osteoclasts, we found that Dock5 localized to adhesion structures: the podosome belt and the sealing zone. Using small interfering RNAs, we performed a partial silencing of Dock5, reducing the protein levels by 70%. This was compatible with osteoclast survival and differentiation and it did not affect the fusion index. Interestingly, Dock5 deficient osteoclasts presented very few podosomes and they were unable to assemble a podosome belt. When plated on mineralized substrates, osteoclasts failed to contract and to organize a sealing zone. Consequently, reduction of Dock5 expression in osteoclasts impaired mineralized matrix resorption. Lower Dock5 expression also resulted in strong reduction of the level of active Rac in osteoclasts. To study the influence of Dock5 on mouse bone density in vivo, Dock5 gene was deleted. Micro-computed tomography analyses of the mice will be discussed. Our observations suggest that Dock5 is a major activator of Rac in osteoclasts and that the exchange factor plays an essential role for adhesion structure organization and then mineralized matrix resorption. As a Rac activator predominantly expressed in osteoclasts, Dock5 represents an interesting new candidate for antiresorptive therapy.

874/B71

Tenascin-C and Tenascin-W in Cancer Stroma: A Role in Tumor Growth and Metastasis?

R. Chiquet-Ehrismann[1], M. Degen[1], F. Brellier[1], C. Rüegg[2], A. Merlo[3]; [1]Friedrich Miescher Institute, Basel, Switzerland, [2]Division of Experimental Oncology, University of Lausanne, Lausanne, Switzerland, [3]Laboratory of Molecular Neuro-Oncology, University Hospital, Basel, Switzerland

Epithelial tissues depend on normal stromal cells and the basement membrane for maintenance of tissue homeostasis. These interactions continue to be important in epithelial pathologies. During carcinogenesis tumor-associated cells of the stromal compartment express pro-proliferative paracrine signals for epithelial cells and stimulate angiogenesis. Therefore, understanding the mechanisms of the complex

crosstalk between the cancerous epithelial cells and the tumor stroma might lead to novel approaches for cancer therapies which target the functions of the activated stromal cells. A prominent extracellular matrix protein specifically present in tumor stroma is tenascin-C (TNC). Interestingly, TNC was shown to be expressed around angiogenic vessels in many tumors and to promote angiogenesis in cell culture studies. Furthermore, TNC added to a fibronectin substratum stimulated cancer cell growth and migration in vitro. Therefore, TNC is a candidate molecule mediating the pro-tumorigenic effects of tumor stroma. Glioblastomas are highly invasive and aggressive brain tumors that show limited response to conventional therapies. We observed in human glioblastoma cells high Notch2 protein levels to coincide with expression of TNC. Expression of activated forms of Notch2 or Notch1 in glioma cells triggered RBPJk-dependent induction of TNC transcription. Furthermore, transfection of activated Notch2 or Notch1 increased endogenous TNC protein production. Since increasing amounts of TNC stimulated glioma cell migration, this may represent a mechanism for the invasive properties of glioma cells controlled by Notch and defines a novel oncogenic pathway in gliomagenesis to be targeted for therapeutic intervention in glioblastoma patients. Recently, we determined the presence of a novel tenascin family member, tenascin-W (TNW), in the stroma of breast and colon cancers and elevated levels of TNW were found in the sera of colon cancer patients. In vitro, TNW did not interfere with cancer cell adhesion to fibronectin, but promoted migration of breast cancer cells towards fibronectin. These data imply that TNW expression in the activated tumor stroma facilitates tumorigenesis by supporting the migratory behavior of cancer cells.

875/B72

Invadopodia Activity Is Promoted by Extracellular Matrix Rigidity.

K. Branch[1], N. Alexander[1], A. Parekh[1], E. Clark[2], I. Iwueke[1], S. Guelcher[3], A. Weaver[1,2]; [1]Cancer Biology, Vanderbilt University, Nashville, TN, [2]Pathology, Vanderbilt University, Nashville, TN, [3]Chemical Engineering, Vanderbilt University, Nashville, TN

Invadopodia are actin-rich subcellular protrusions with associated proteases used by cancer cells to degrade extracellular matrix (ECM). Despite close contact with ECM substrates, it is unknown whether physical or chemical ECM signals regulate invadopodia function. Here, we report that ECM rigidity directly increases both the number and activity of invadopodia. Transduction of ECM rigidity signals depends on the cellular contractile apparatus, as inhibition of nonmuscle myosin II abrogates invadopodia-associated degradation. Whereas myosin IIA, IIB, and phosphorylated myosin light chain do not localize to invadopodia puncta, active phosphorylated forms of the mechanosensing proteins p130Cas (Cas) and focal adhesion kinase (FAK) are present in actively degrading invadopodia and the levels of phospho-Cas and phospho-FAK in active invadopodia are sensitive to myosin inhibitors. Overexpression of Cas or FAK further enhances invadopodia activity in cells plated on rigid polyacrylamide substrates. Thus, in invasive cells, ECM rigidity signals lead to increased matrix-degrading activity at invadopodia, via a myosin II-FAK/Cas pathway. These data suggest a potential mechanism, via invadopodia, for the reported correlation of tissue density with cancer aggressiveness.

876/B73

The Importance of the Intracellular Glutathione Pool of Rape Cells Exposed to Heavy Metal Stress.

K. Viehweger, G. Geipel; Institute of Radiochemistry, Forschungszentrum Dresden, Dresden, Germany

Uranium is a widespread radioactive toxic heavy metal, released into the biosphere mostly by military purposes and nuclear industry. It is taken up by plant root systems and its chemical toxicity is much more dangerous than the radiological. Thus cell suspensions of rape (*Brassica napus*) revealed similar intracellular defence reactions after uranium exposure like it is described for other heavy metals (1). Glutathion is one of the key players in this network, because of its ability to complex xenobiotics via the action of glutathione-S-transferase, its redox-capacity, and/or as precursor in the biosynthesis of heavy metal-binding peptides, e.g. phytochelatines. Rape cells react with a decrease of the cytoplasmic glutathione pool, revealed by HPLC and TLC, respectively. Time-resolved laser-induced fluorescence spectroscopy (TRLFS) measurements gave an insight in interactions between glutathione and uranium. The possible reduction of the soluble uranium(VI) to an insoluble oxidation state of (IV) by glutathione can be excluded, because of lacking uranium(IV) in the cytoplasm, detected by photoacustic measurements. These findings and the time course of dropping the glutathione pool suggest an involvement in the biosynthesis of pytochelatines, which is proven by HPLC. Experiments addressing the physiological relevance of these parts of cellular defence mechanisms are under way, especially to elucidate whose impact on intracellular heavy metal sequestration. (1) Clemens, S. (2001). "Molecular mechanisms of plant metal tolerance and homeostasis." Planta V212 (4): 475-486.

877/B74

Uranium Speciation after Contact with Plant Cells.

G. Geipel, K. Viehweger, G. Bernhard; Institute of Radiochemistry, Forschungszentrum Dresden, Dresden, Germany

Uranium is an ubiqutous element. Besides this depleted uranium amunition as well as uranium mining and milling and manifold other use of uranium leads to an increase of uranium contamination in the environment. Application of laser-induced and time-resolved methods allow the direct determination of uranium speciation at extremely low concentrations. This behaviour can be directly observed due to the properties extraorbitant luminescence properties of uranium-(VI) and the photoacoustic detection of uranium-(IV). The uranium-(VI) luminescence can be excitet around 410 nm, whereas the photoacoustic spectra of uranium-(IV) where observed in the wavelength range 620 nm to 680 nm. Contact of dissolved uranium with living cells at ambient conditions changes dramatically the uranium speciation. Some examples fluorescence properties of uranium species relevant to the environment are shown. The change of this speciation can be observed then due to a change in luminescence properties. Besides of several organic phosphate binding forms although other uranium species were found as uranium bond to phenolic and thiol groups. Some of them do not emit any luminescence at room temperature. Nevertheless low temperature measurements allow the assignment of species not fluorescing at room temperature, due to strong dynamic quench effects of H_2O molecules and COO^- groups.

878/B75

The Effect of Gold Nanorods on Cardiac Fibroblast-Mediated Collagen Gel Contraction.

P. N. Sisco[2], C. G. Wilson[1], E. Mironova[1], S. Baxter[3], C. J. Murphy[2], E. Goldsmith[1]; [1]Cell Biology and Anatomy, Univ. of South Carolina,

Columbia, SC, [2]Chemistry and Biochemistry, Univ. of South Carolina, Columbia, SC, [3]Dept. of Mechanical Engineering, Univ. of South Carolina, Columbia, SC

Cell behavior in the presence of nanomaterials is typically explored through simple viability assays, but there is mounting evidence that nanomaterials can have more subtle effects on a variety of cell functions. Numerous studies have documented the cellular uptake and cytotoxicity of gold nanoparticles in different cell types, but very little is known about how nanoparticles affect cellular function. In this work, we examine how cardiac fibroblast-mediated extracellular matrix remodeling is perturbed by gold nanorods. Gold nanorods (392 nm long x 22 nm wide) were prepared in aqueous solution using a seed-mediated surfactant-directed approach and overcoated with polystyrene sulfonate. To investigate the capacity for nanomaterials to modulate this process, 3-D collagen gel constructs were prepared from neonatal cardiac fibroblasts and type I collagen with and without gold nanorods. Over a 24h period the collagen-nanomaterial composite scaffolds showed significantly less contraction than controls. The reduced gel contraction observed in the presence of polymer coated gold nanorods was not due to cell death, as LDH and confocal analysis demonstrated no difference in fibroblast viability between control and rod-treated gels. The exposure of cardiac fibroblasts to gold nanorods did alter fibroblast phenotype, as evidenced by an upregulation of beta-actin and a down regulation of alpha-smooth muscle actin and collagen type I mRNA. Taken together, these data indicate that biocompatible nanomaterials have the capacity to regulate fibroblast-mediated matrix remodeling. Targeted delivery of nanomaterials may represent a novel mechanism for managing pathological cardiac remodeling.

879/B76

The Presence of Biophysical Cues in the Biomimetic Range Differentially Influences the Treatment Response of Squamous Cell Carcinomas and Normal Tissue.

S. Liliensiek[1], S. Huang[2], P. Harari[2], P. F. Nealey[3], C. J. Murphy[1]; [1]Surgical Sciences, University of Wisconsin, Madison, WI, [2]Human Oncology, University of Wisconsin, Madison, WI, [3]Chemical and Biological Engineering, University of Wisconsin, Madison, WI

A fundamental feature of Squamous Cell Carcinomas (SCC) and other neoplasms is the dysregulated or complete lack of response to specific environmental cues. Work performed in our laboratories and others have demonstrated that basement membranes (BMs) possess biophysical cues in the form of a complex three-dimensional topography (fibers, pores and elevations) in the 20-400 nm range. Additionally, BMs possess an intrinsic compliance. The impact of topographic cues and the effect of substratum compliance on neoplastic cells remains largely undetermined. We have recently conducted studies investigating the impact of topographic cues and sensitization of SCC cells to radiation treatment. Primary keratinocytes and SCC-1483 cells were plated onto substrates containing chemically identical planar control surfaces or surfaces patterned with 400 nm, 1400 nm or 4000 nm pitch topographies. 24 hours post-plating, cells were treated with 0, 3, 6 or 9 Gy of irradiation using a 137Cs irradiator. Cells were allowed to recover for 24 hours prior to analysis using both apoptosis and DNA damage. Both early (Annexin V alone) and late (Annexin V + Propidium Iodide) apoptosis was detected by flow cytometry where we observed a 3-fold increase in early apoptosis of the SCC1483 cells. In contrast, no difference in early or late apoptosis was observed in the primary keratinocytes. Results from our initial experiments with labeling of damaged phosphorylated histone H2AX (γ-H2AX) foci indicate that the topographically patterned features influence the DNA damage profile following radiation. We observed a scale effect with the greatest number of γ-H2AX foci on the 4000 nm pitch. In summary, we found that the presence of topographic cues differentially affects the response to radiation treatment. Defining the biophysical characteristics of the microenvironment of SCC will contribute to our fundamental understanding of the consequences of neoplastic transformation and potentially lead to the development of enhanced cancer treatments.

880/B77

The Transition of Cellular Behaviors Between Biomimetic-Scale and Micro-Scale Topographically Patterned Substrates.

C. J. Murphy[1], J. Gasiorowski[1], S. Liliensiek[1], P. Russell[1], P. F. Nealey[2]; [1]Surgical Sciences, University of Wisconsin, Madison, WI, [2]Chemical and Biological Engineering, University of Wisconsin, Madison, WI

Native basement membranes possess an information-rich surface topography composed of interwoven fibers, bumps and pores in the nano-submicron size scale. Mimicking this topographic rich environment for in vitro experiments has been technically challenging. However, our laboratory has fabricated anisotropically grooved surfaces that span the full range of nano- to micron-scale dimensions of topographic features found in vivo. These surfaces have become valuable tools to design reductionist experiments in order to test how the size scale of an individual physical feature type influences cell behaviors. While it is intuitive that cells would behave differently on topographically rich versus flat surfaces, our investigations have also uncovered a menu of cell behaviors that are modulated as the size-scale of specific topographic features is altered. Cell behaviors are differentially modulated on substrates possessing features with biomimetic (nano-submicron) size scales compared to surfaces patterned with features in the micron size-scale. Specifically, a distinct transition in cell behavior is observed over the approximate range of 600 nm-800 nm feature dimensions. Cells behave differently on surfaces patterned with features of smaller (biomimetic) dimensional values than this transition zone than on features larger than this transition zone. These trends in the data suggest that cells can sense a "transition zone" in size-scale. A size-scale dependent transition zone was identified for cell adhesion, migration, proliferation, and some aspects of differentiation. In contrast, alignment for most cells appears relatively insensitive to feature scale. These findings add to our understanding of how biophysical cues delivered from the extracellular matrix contribute to the maintenance of homeostasis and participate in pathophysiological processes.

881/B78

Role of Extrahepatic Stellate Cells (Vitamin A-storing Cells) in the Rat.

H. Senoo, M. Miura, K. Yoshikawa, Y. Mezaki, K. Imai, N. Yamaguchi; Cell Biology and Histology, Akita University School of Medicine, Akita, Japan

Objective: Extrahepatic stellate cells have been reported in mammals including human and in lower vertebrates. Roles of these cells have not yet been thoroughly investigated. To examine the roles of extrahepatic stellate cells in the rat intestine, we performed this study. Methods: Vitamin A (retinyl palmitate) was administered to rats through a stomach tube. Autofluorescence of vitamin A was chased by fluorescence microscopy. Vitamin A in each organ and serum was quantified by high-performance liquid chromatography. Cells in the lamina propria

mucosae in the digestive tract were characterized by immunofluorescence for desmin and alpha-smooth muscle actin, specific markers for the hepatic stellate cells, and electron microscopy. Results: Vitamin A was absorbed from absorptive epithelial cells in the intestine. Absorbed vitamin A was transferred to mesenchymal cells (mainly fibroblastic cells) in the lamina propria mucosae in the intestine and then to the liver. Vitamin A, once stored in the liver, spilled out from the liver, circulated in the plasma was taken up and stored in fibroblastic cells in the lamina propria mucosae. These cells emitted autofluorescence of vitamin A, stored many lipid droplets in the cytoplasm, and expressed desmin and alpha-smooth muscle actin. Conclusions: These data suggest that these fibroblastic cells are extrahepatic stellate cells in lamina propria mucosae of the digestive tract and that these cells have potentiality of vitamin A storage in rats.

882/B79

Methods for Three-Dimensional Extracellular Matrix Ultrastructure and Cellular Response Characterization of Arteries Pre- and Post-Stenting using Serial Block Face Scanning Electron Microscopy.

S. Saatchi[1], J. Buchanan[2], A. J. Bernstein[3], C. Genoud[4], C. Xu[5], S. Schrepfer[6], S. J. Smith[2], D. P. Lee[7], P. G. Yock[1], C. A. Taylor[1];
[1]Bioengineering, Stanford University, Stanford, CA, [2]Molecular and Cellular Physiology, Stanford University, Stanford, CA, [3]Electrical Engineering, Stanford University, Stanford, CA, [4]Gatan, Inc., Pleasanton, CA, [5]Vascular Surgery, Stanford University, Stanford, CA, [6]Cardiothoracic Surgery, Stanford University, Stanford, CA, [7]Cardiovascular Medicine, Stanford University, Stanford, CA

The current understanding of the three-dimensional (3D) cellular responses and vessel wall microstructural disruptions that occur in response to stent treatment is quite limited. We describe novel methods to enable the use of 3D high resolution Serial Block Face Scanning Electron Microscopy (SBFSEM) to investigate 3D vessel ultrastructure and response to stents. SBFSEM images resin-embedded sample block faces, which precludes the possibility of on-section contrast enhancing staining. Highly contrasted specimens are required to visualize smooth muscle cells (SMC) and elastin with SBFSEM. Therefore, we developed heavy metal en bloc combination staining techniques, along with image analysis methods, to investigate 3D medial ultrastructure and cellular responses to stents using SBFSEM. Adult Sprague-Dawley rats were used for control and stented groups with coronary bare metal stent implantation in the aorta. At 3 weeks post-implantation, 1-2mm thick samples were harvested along the aorta. Ten en bloc staining methods were developed and evaluated using transmission electron microscopy and selective SBFSEM (Gatan, Inc). A preliminary image analysis algorithm, including image processing, automated segmentation, quantitative analysis, and 3D rendering, was developed to analyze the initial SBFSEM images (Matlab, EM3D). Two best en bloc staining methods were selected. Method 1 (Osmium imidazole, 5% tannic acid, 4% uranyl acetate en bloc stain) provided SBFSEM SMC contrast, enabled automated SMC segmentation and volume quantification, as well as 3D SMC renderings. Method 2 (2% OsO4, 5% tannic acid, 1% PdCl en bloc stain, 5% uranyl acetate en bloc stain) yielded sufficient SBFSEM contrast for both elastin and SMCs. Preliminary results suggest observable increases in elastin disorganization and SMC content with stent implantation. In conclusion, we developed optimal en bloc staining methods for SBFSEM identification of SMCs and elastin, an automated SMC analysis algorithm, and 3D depictions of elastin and SMCs. Renderings now enable 3D investigation of ultrastructural organization, cell shape, cell-cell interaction, and cell-extracellular matrix interaction to improve the understanding of stent impact on the vessel wall.

883/B80

Tenascin-C Induces Epithelial Mesenchymal Transition-Like Change through Recruitment Of αvβ6 Integrin and Phosphorylation of Focal Adhesion Kinase by Src in Breast Cancer Cells.

T. Yosida, K. Nagaharu, k. Imanaka-Yoshida; Pathol & Matrix Biol, Mie University Sch Med, Tsu, Japan

Tenascin-C (TN-C) is an adhesion modulatory extracellular matrix glycoprotein that is highly expressed in most solid tumors. Higher TN-C expression is correlated with poorer outcome in patients with some cancers including breast cancer. Epithelial mesenchymal transition (EMT) is a key step of tumor invasion and metastasis. Here, we examined whether TN-C induces EMT events in breast cancer cell line MCF-7. Addition of TN-C to the medium leaded to internalization of E-cadherin and β-catenin, followed by EMT-like morphological change. Supplementary TGF-β1 treatment showed translocation of β-catenin into the nucleus. The morphological change was blocked by adding a neutralizing antibody of integrin αv or src kinase inhibitor. Immunofluorescent microscopy and Western blotting demonstrated that expression and recruitment of αvβ6 integrin was upregulated in the cells showing EMT-like phenotype. Further, EMT change was closely correlated with phosphorylation of focal adhesion kinase (FAK) at Y861 and Y925 of src kinase substrate sites. The phosphorylation was co-localized with β6 subunit-positive adhesion plaques. TN-C could induce EMT-like phenotype in breast cancer cells through recruitment of αvβ6 integrin, src activation and FAK phosphorylation, indicating to be responsible for promotive effects on progression of breast cancer.

884/B81

Tenascin-C May Accelerate Adverse Cardiac Remodeling and Cardiac Failure.

K. Imanaka-Yoshida, T. Nishioka, Y. Nagano, R. Hida, T. Yoshida; Pathology and matrix Biology, Mie University, Tsu, Japan

Tenascin-C (TNC) is an extraceulluar matrix glycoprotein, with strong bioactivity, transiently expressed associated with tissue remodeling during embryogenesis, injury, inflammation,wound healing or cancer invasion. TNC is sparsely expressed in healthy adult heart but markedly upregulated under various pathological condition such as myocardial infarction(MI), myocarditis or dilated cardiomyopathy(DCM). Recently, we reported that the MI or DCM patients with high serum level of TNC have a greater incidence of ventricular remodeling and worse prognosis. The aim of the present study was to examine whether TNC accelerates adverse ventricular remodeling. Myocardial infarction(MI) was produced by ligating the coronary artery in BALB/c background 10- to 12-wk-old male TNC knockout (TNKO) mice and sibling wild type (WT) mice. In WT mice, TNC was expressed at border zone between intact myocardium and infarct lesion within 24 hours after ligation, peaked around day 3-5, then down-regulated and disappeared by day 28. The morphometrically determined infarct size and survival rate on day 28 were comparable between the WT+MI and TNKO+MI groups. Echocardiograph and hemodynamic analysis demonstrated no significant differences in LV size and function between sham operated KO and WT. LV end-diastolic diameter was significantly increased accompanied with reduced ejection fraction in WT+MI mice to compare with WT+sham mice. Myocardial stiffness and LV end-diastolic pressure were also increased in WT+MI mice. In KO+MI mice, LV end-diastolic diameter, myocardial stiffness and LV end-diastolic pressure was significantly increased to compare with KO+sham mice. However, end-diastolic pressure, dimension, and

myocardial stiffness in KO+MI were lower than those in WT+MI. Histological examination showed that tissue healing appeared to proceed normal and no distinct difference in collagen fibers formation in scar tissue. However, quantitative analysis demonstrated interstitial fibrosis in the residual myocardium adjacent to infarcted area was significantly lower in KO+MI to compare with WT+MI. TNC may accelerate ventricular remodeling, cardiac failure and fibrosis in residual myocardium after MI.

885/B82

Laminin Peptide C16 Regulates Migration, Invasion and Protease Activity of Adenoid Cystic Carcinoma Cells through Integrins, AKT and ERK.

L. Gama-de-Souza, V. Freitas, R. Jaeger; Departament of Cell and Developmental Biology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo SP, Brazil

We studied induction of migration, invasion and protease activity by laminin-derived peptide C16 (KAFDITYVRLKF, gamma1 chain) in a cell line (CAC2) derived from adenoid cystic carcinoma, a malignant salivary gland tumor. Laminin gamma1 was immunolocalized in adenoid cystic carcinoma "in vivo" and "in vitro". C16 increased migratory activity of CAC2 cells, as shown by monolayer wound assay, Transwell migration assay and time-lapse video microscopy. This peptide also stimulated cell invasion in Transwell chambers coated with Matrigel. Invasion depends on protease activity. Zymograms showed that C16 increased secretion of MMPs 2 and 9. Different signaling pathways could be related to C16 regulation in CAC2 cells. Immunoblot showed that C16 increased phosphorylation of both AKT and ERK compared to controls. To study putative receptors of this peptide we used affinity chromatography. Membrane preparations were run through C16-affinity columns. A 40kDa band was eluted and analyzed by mass spectrometry (LC-MS/MS) identifying a collagen alpha1 chain. The collagen fragment eluted could be part of a protein complex involving C16. This protein complex may include integrins, which are collagen receptors. CAC2 cells exhibited alphaV, alpha5, beta3 and beta1 integrins. siRNA knockdown of these integrins inhibited both C16-induced migration and protease activity. We propose that C16 increases migration, invasion and protease activity of a human salivary gland adenoid cystic carcinoma cell line through alpha5beta1 and alphaVbeta3 integrins. The signal generated by C16 is transduced by AKT and ERK1/2 signaling pathways. Support: The State of São Paulo Research Foundation (FAPESP, 2006/57079-4), and Brazilian Research Council (CNPq, 304868/2006-0 and 470622/2007-5).

886/B83

Effects of Successive Radiofrequency Treatments on the Fibroblast of the Dermis in Sprague-Dawley Rats.

A. Zuasti[1], E. Beltrán-Frutos[1], C. Ferrer[1], S. Navarro[3], V. Seco-Rovira[1], M. Canteras[2], L. Pastor[1]; [1]Cell Biology and Histology, University of Murcia, Murcia, Spain, [2]Department of Statistics, University of Murcia, Murcia, Spain, [3]Group Tahe, Magaña Distributions, Murcia, Spain

The effects produced by radiofrequency on connective tissue cause modifications in the fibroblast. Until now, no histological evaluation has been made of the changes that take place after successive applications of low power radiofrequency. This work studies such changes in the fibroblast, looking at the degree of fibroplasia and biosynthetic activity. In the experiment 70 (50 treated and 20 control) 2 month-old Sprague-Dawley rats were divided into four groups, which were subjected to 1, 2, 3 or 4 sessions of radiofrequency in the tail. The various groups were sacrificed after 3, 6, 9 or 12 weeks. Fibroblasts per unit area were determined in all the animals. The fibroblasts per unit area positive to Heat Shock Protein (HSP-47) were assessed by immunohistochemical methods. Statistical evaluation showed that: a) in animal group with a single treatment the above variables studied to twelve weeks showed the same values as the controls b) a single treatment after 3 weeks increased the values of the variables c) a second and third treatment every three weeks prolonged the effect of radiofrequency beyond the ninth week. In conclusion, the treatment applied three and six weeks after the first one increases the activity of fibroblast. Financed by Project SUE-0C 06/01-0004 of Consejería de Educación y Cultura CARM, and Fellow of Esther Beltran-Frutos 07900/BPS/07.

887/B84

Regulation of DNA Double-Strand Break Repair Genes and Processes by Extracellular Matrix Signaling.

L. B. Gentile[1], E. R. Fraley[1], D. Nahar[2], A. Rizki[1]; [1]Radiation Oncology, Virginia Commonwealth University (VCU), Richmond, VA, [2]Life Sciences Division, Ernest Orlando Lawrence Berkeley National Laboratory, Berkeley, CA

The extracellular microenvironment (ECM) can have significant effects on gene expression. The purpose of this study was to compare gene expression levels between breast epithelial cells grown in different microenvironments, with particular emphasis on DNA repair genes because of their importance in maintaining genome stability and cancer avoidance. We asked how ECM signaling altered expression of genes that are relevant to DNA double-strand break repair using a series of cell lines HMT-3522 containing non-invasive S1, pre-invasive S2, S3-A, S3-B, S3-C, and invasive T4-2 cells. Using cDNA microarrays and verifying expression differences by RT-PCR we found that ADPRT and RAD50 were more highly expressed in three-dimensional laminin-rich ECM, 3DlrECM, cultures than in monolayer 2D; 53BP2 had lower expression in 3DlrECM than in 2D. For further analysis, we focused on the MRN complex proteins MRE11, RAD50, and NBS1. In the non-invasive S1 cell lines, we found that progressive passaging of cells, growth, cell-cell contact, and signaling via the β1 integrin receptor play a role in the co-regulation of the levels of these proteins by lrECM. For the levels of MRE11 in 2D cultures we did not find any difference among the breast cancer progression model S1, S2, S3-A, S3-B, S3-C and T4-2. However when we compared the levels of MRE11 in 2D cultures to those in 3D cultures the levels of this protein were increased 2.8 fold in S1 cells, 1 fold in S2 cells, 1.8 fold in S3-A cells, 1.9 fold in S3-B, 1.7 in S3-C and 1.5 in T4-2 cells. When S3-C were cultured in 2D cultures the levels of NBS1 and RAD50 were increased compared to the other cell lines. To support the functional significance of the MRN complex gene expression regulation by ECM, we found that the kinetics of MRE11 foci formation at the sites of DNA double-strand breaks after irradiation of S1 cells are regulated by β1 integrin-mediated ECM signaling. In conclusion, we showed that lrECM regulates the levels of expression of genes that are important in DNA double-strand break repair, adding a new class of genes regulated by microenvironmental cues, and are determining the functional significance of this for DNA double-strand break repair and genome stability.

888/B85

Engineering Strategies to Recapitulate Epithelial Morphogenesis Using Natural and Synthetic Three Dimensional Matrices.

Y. A. Miroshnikova[1,2], C. Frantz[2], J. L. Leight[3], K. Johnson[3], D. M. Jorgens[4], M. Auer[4], L. Spirio[5], A. Sieminski[1], V. M. Weaver[2,3]; [1]Olin College of Engineering, Needham, MA, [2]Surgery and Center for Bioengineering and Tissue Regeneration, University of California San Francisco, San Francisco, CA, [3]Bioengineering and Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA, [4]Lawrence Berkeley National Laboratory, University of California, Berkeley, CA, [5]PuraMatrix/3DM Inc., Cambridge, MA

Epithelial tissue morphogenesis proceeds within the context of a three dimensional (3D) extracellular matrix (ECM). Accordingly, to clarify the molecular basis of tissue-specific differentiation and disease, a variety of 3D systems exploiting natural ECMs have been developed, such as reconstituted basement membrane (rBM) and purified collagen hydrogels. These natural hydrogels recapitulate epithelial tissue architecture and behaviors in vitro with reasonable fidelity. Nevertheless, natural matrices suffer from considerable preparation variability and remain poorly defined biochemically and biophysically. In addition, the methods to study and manipulate epithelial cell behavior in 3D, as well as the definition of 3D, vary appreciably between and even within laboratories. To understand epithelial cell biology requires defined biomaterials in which biochemical, topological and biophysical properties can be systematically varied. Towards this goal we used the nonmalignant MCF10A mammary epithelial cell (MEC) line and conducted a systematic analysis of acinar morphogenesis using the natural hydrogels collagen type I and rBM and three synthetic matrices: rBM-conjugated poly acrylamide gels, self assembling peptide gels (PuraMatrix) with and without rBM and hyaluronic acid gels with and without rBM. We assessed acini formation, cell growth and death and colony integrity and heterogeneity of MECs either fully embedded within these natural and synthetic gels compared to those receiving a matrix overlay or pseudo 3D matrix cue. All biomaterials supported acinar morphogenesis and yielded viable polarized, growth-arrested MEC structures with cell-cell adherens junctions and deposition of endogenous BM proteins, however, optimal growth control, survival and lumen formation, as well as colony integrity and homogeneity were observed when cells were completely embedded within the ECM and when ECM remodeling was permitted. Consequently, optimal acinar morphogenesis was observed using rBM, collagen I and PuraMatrix. Rigorous, morphometric and quantatitive analysis as well as immunohistochemistry, and electron microscopy are in progress. (Supp: NIH 7R01CA078731-07, DOD W81XWH-05-1-330, CIRM RS1-00449, and DOE A107165 to VMW).

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Tissue-Engineered Human Extracellular Matrix as a Biological Substrate for Regenerative Medicine and Bioengineering Applications.

M. Zimber, F. Zeigler, E. Pinney, G. Naughton, R. Kellar; Histogen Inc., San Diego, CA

Extracellular matrix (ECM) in the developing embryo supports rapid cell proliferation and healing without the formation of scars or adhesions. Such an ECM could prove to have therapeutic benefits in tissue growth and regeneration. We designed a cell culture system to mimic the early embryonic environment prior to angiogenesis (hypoxia and reduced gravitational forces) to grow human neonatal fibroblasts in three-dimensions that generated a human extracellular matrix (hECM) and soluble proteins with fetal-like properties. DNA microarray analysis showed the differential expression of over 5000 genes under the hypoxic versus traditional, normoxic tissue culture conditions. The hECM produced was similar to fetal mesenchymal tissue in that it was rich in collagens type I, III, IV, and V, and glycoproteins such as fibronectin, SPARC, thrombospondin, and hyaluronic acid. Hypoxic culture conditions induced the expression of hypoxia-inducible factor 1a and also enhanced the expression of wound healing and organogenesis growth factors such as VEGF and TGFb. The hECM supported the growth and maintenance of undifferentiated pluripotent human embryonic stem cells and provided an appropriate cellular substrate for wound healing in damaged tissues. In addition, we have successfully used the hECM as a surface coating on mesh materials, such as nylon and polypropylene. The hECM acts as a biological interface and improve biocompatibility for biomedical devices, including stents and inert vascular grafts, where modulation of the healing response can help in end-device function. These results support a range of tissue regeneration applications for this bioengineered hECM that include acting as a wound healing matrix, providing a substrate and delivery vehicle for cell-based therapies, and producing a biological surface coating for biomedical devices and implants.

890/B87

L- and S-endoglin Differentially Modulate Biological Proprties in L6E9 Myoblasts.

S. Velasco, M. Pericacho, J. M. López-Novoa, A. Rodriguez-Barbero; Fisiología y Farmacología, Universidad de Salamanca, Salamanca, Spain

Transforming growth factor beta (TGFβ) regulates cellular processes by binding to type I (ALK, activin like kinase) and type II TGFβ receptors. In addition to these signalling receptors, endoglin, a transmembrane glycoprotein, acts an auxiliary receptor for TGFβ. There are two different alternatively spliced isoforms of endoglin, L-endoglin (L, long) and S-endoglin (S, short). We previously reported L-endoglin enhanced the ALK1-Id1 pathway, while S-endoglin promoted the ALK5-PAI1 route. These effects on signalling are supported by biological effects of TGFβ1 induced collagen I expression and inhibition on cell proliferation. There are many studies about the effect of L-endoglin on cellular properties, however, little is known about the effects of S-endoglin expression. The aim of this study was to investigate the effect of S-endoglin expression on the biological properties of L6E9 myoblasts. L6E9 myoblasts were transfected with L- and S- endoglin or with the empty vector (Mock). Endoglin transfection was assessed by western blot, PCR, and immunofluorescence. We analyzed the effects of L-and S-endoglin on extracellular matrix synthesis by western blot, cell proliferation by MTT, cell cycle by flow citometry and expression of cyclin D1 by western blot. Cyclooxigenase-2 (COX-2) expression was analyzed by western blot and PCR, and the activity of this enzyme by promoter assays and prostaglandin E2 (PGE2) ELISA. We found that while L-endoglin expression decreased collagen I and CTGF (connective tissue growth factor) and increased proliferation respect to Mock cells, S-endoglin strongly increased collagen I and CTGF expression and reduced cell proliferation. Interestingly, S-endoglin enhanced TGFβ-induced expression, COX-2 activity and PGE2 production while L-endoglin reduced these responses. Our data demonstrate a different and sometimes opposed effect of L and S isoforms of endoglin on the regulation of biological properties in L6E9 myoblasts.

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Evidence of Durotaxis in Transformed Mammary Epithelial Cells.

J. Lopez, V. Weaver; UCSF, San Francisco, CA

The movement of cells in the direction of a stiffness gradient termed durotaxis has been demonstrated in recent years in fibroblasts and vascular endothelial cells. Here we present evidence that transformed mammary epithelial cells (MECs) respond to durotactic gradients. We explore this phenomenon in a 2D environment using gels of precisely calibrated stiffness as well as gels demonstrating a gradient of stiffness. MECs migrating along stiffer matrices display increased speed while also moving in the direction of stiffer substrates. We generated stiffness maps of mammary gland tumors derived from MMTV-PyV mT transgenic mice using atomic force microscopy. We found that the area surrounding the tumor vasculature and the invading front of the tumors are stiffer than the surrounding tissue. These areas may serve as a directional route by which transformed epithelial cells exit away from the primary site.

Exocytosis: Plasma Membrane Events

892/B91

Involvement of ELKS, an Active Zone Protein, in Exocytotic Release in Mast Cells.

H. Nomura, N. Hirashima; Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan

The active zone is a specialized region of the presynaptic plasma membrane where the neurotransmitter release occurs. Several proteins specific to the active zone have been reported, including Munc13, RIM1 (Rab3 interacting molecule 1), bassoon, Piccolo/Aczonin, and ELKS/CAST (CAZ-associated structural protein). Among these proteins, ELKS plays a critical role in the formation of the active zone. Indeed, when Bruchpilot, homolog to ELKS is decreased by RNAi, T bars of active zones disappeared. We have studied the mechanism of exocytosis and revealed the involvement of SNARE (soluble N-ethyl maleimide-sensitive factor attachment protein receptor) proteins in exocytotic release in mast cells which are responsible for allergic responses. Furthermore, we demonstrated that Munc13-1, one of active zone proteins, regulates exocytosis of mast cells. These results suggest that the exocytotic machinery in mast cells is similar to that in neuronal cells and implicate that mast cells have an active zone like structure. Here, we show that ELKS is involved in exocytotic release in mast cells. We detected the expression ELKS in mast cells by RT-PCR and Western blotting. To analyze the role of ELKS in exocytotic release, we investigated the effects of expression level of ELKS on secretion. Knockdown of ELKS by RNAi caused decrease in secretion without affecting Ca^{2+} mobilization, whereas overexpression of ELKS enhanced the secretion, indicating that ELKS is a positive regulator of exocytosis. Immunocytochemical experiments revealed the translocation of ELKS from the cytoplasm to the subplasmalemmal region when mast cells are activated through FcεRI. Similar results were obtained in the cells that express YFP-tagged ELKS. These results suggest that ELKS facilitates exocytosis in mast cells, translocating from the cytoplasm to the subplasmalemmal region upon stimulation.

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Manipulations of Intact Sea Urchin Eggs In Vitro: a New Paradigm for Molecular Studies of Regulated Membrane Fusion.

T. Rogasevskaia[1], J. R. Coorssen[1,2]; [1]Dept Physiology & Biophysics, University of Calgary, Calgary, AB, Canada, [2]Dept Molecular Physiology, University of Western Sydney, Sydney, NSW, Australia

Studies using isolated sea urchin cell surface complexes (CSC) and cortical vesicles (CV) have proven invaluable in dissecting the molecular mechanisms underlying docking and Ca2+-triggered membrane fusion [1-3]. A limitation has been that only acute molecular manipulations are possible in isolated CSC & CV in vitro. We have developed protocols enabling longer-term manipulations more akin to those associated with mammalian cell culture to further identify critical components of triggered fusion. Intact eggs of S. purpuratus were incubated at 7°C for 20h in Ca2+-free artificial sea water (ASW) with selective reagents, targeting endogenous metabolic pathways. Following incubations, control eggs were able to raise fertilization envelopes upon addition of Ca2+ -ionophore to the ASW (e.g. CV undergo triggered vectorial release). CV isolated from treated eggs were subjected to standard fusion assays and analyses of membrane components. Initial data indicate that CV membrane fusion is ultimately affected by treatment of eggs with simvastatin and atorvastatin, competitive inhibitors of HMG-CoA reductase, and the squalene synthase inhibitor, zaragozic acid. Treatment of eggs with these cholesterol (CHOL) lowering agents caused significant inhibition in the extent, Ca2+sensitivity and kinetics of CV fusion, correlating with decreased CHOL levels. However, isolated CV fully recovered all fusion parameters when supplemented with exogenous CHOL, and showed selective recovery of the ability to fuse when treated with α-tocopherol. Results are consistent with findings concerning critical roles of these molecules and their inherent negative curvature in the fusion mechanism [2,3]. Treatment with D609, a phospholipase C inhibitor, yielded reduced CV phosphatidylethanolamine (PE) levels, correlating with decreased fusion extent and kinetics, again consistent with a role for PE in the fusion pathway [3]. The data thus indicate that these longer-term manipulations enable effective and meaningful molecular alterations that aid further dissection of the docking and membrane fusion stages, providing new directions for exocytosis research. 1) Biochimie (2000) 82, 303; 2) J. Cell Sci.(2005) 118, 4833; 3) Biophys. J.(2008) 94, 3976.

894/B93

Chronic Exposure to High Glucose Inhibits Insulin Secretion through Perturbation of Pancreatic β-Cell Lipid Rafts.

S. Somanath[1], S. Barg[2], C. Marshall[1], C. Silwood[3], M. Turner[1]; [1]Diabetes and Metabolic Medicine, Barts and The London School of Medicine and Dentistry, London, United Kingdom, [2]Medical Cell Biology, Uppsala University, Uppsala, Sweden, [3]Applied Science, London South Bank University, London, United Kingdom

Objective: Diabetes is characterized by high circulating concentrations of glucose. Among the numerous pancreatic β-cell genes that are modulated by glucose is 3-hydroxy-methylglutaryl coenzyme A reductase, a key enzyme in the biosynthesis of cholesterol. Although high plasma concentrations of cholesterol have deleterious effects, cholesterol performs a number of essential functions within cells, including the stabilization of membrane microdomains. We investigated the effect of high glucose upon insulin secretion, β-cell cholesterol content, and syntaxin 1A association with lipid rafts. **Methods:** Through use of discontinuous density fractionation protocols we have isolated detergent insoluble lipid raft microdomains from INS-1 cells. Cholesterol content was determined using commercial kits and flotillin and syntaxin 1A by Western blotting. Syntaxin 1A clustering and secretory granule-association was determined by TIRF. **Results:** Raft fractions contain characteristically high levels of both cholesterol and flotillin. However, following 3-day exposure of INS-1 cells to 28mM glucose we

observed ~ 30% reduction in the amount of both total cellular cholesterol and membrane cholesterol. This inhibition in membrane cholesterol resulted in a dramatic redistribution of flotillin away from detergent-insoluble rafts, and crucially, this was paralleled by a similar redistribution of syntaxin 1A, the t-SNARE responsible for tethering insulin secretory granules to the plasma membrane at active sites of exocytosis. **Conclusion:** We conclude that, in INS-1 pancreatic β-cells, high glucose-mediated inhibition of cholesterol biosynthesis perturbs lipid raft microdomain stability, resulting in redistribution of syntaxin 1A away from exocytotic fusion sites and an inhibition in insulin secretion.

895/B94

Sec6p Anchors the Exocyst Complex at Sites of Secretion.

J. A. Songer, M. Munson; Biochemistry & Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA

The exocyst is an essential protein complex required for targeting and fusion of secretory vesicles to sites of exocytosis at the plasma membrane. To study the function of the exocyst complex at the molecular level, we performed a structure-based mutational analysis of the *Saccharomyces cerevisiae* exocyst subunit Sec6p. Two "patches" of highly conserved residues are present on the surface of Sec6p; mutation of either patch does not compromise the stability of the protein. Nevertheless, replacement of the wild-type Sec6p with the patch mutants results in severe temperature-sensitive growth and secretion defects. At non-permissive conditions, although trafficking of secretory vesicles to the plasma membrane is unimpaired, none of the exocyst subunits are polarized at sites of secretion. This is consistent with data from other exocyst temperature sensitive mutants, which disrupt the integrity of the complex. Surprisingly, however, these patch mutations result in mislocalized exocyst complexes that remain intact. Our results indicate that assembly and polarization of the exocyst are functionally separable events, and that the conserved patches on the surface of Sec6p are required to anchor exocyst complexes to sites of secretion.

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Regulation of the Entire Rab4 Recycling Pathway by a Single Transmembrane Cargo.

G. Yudowski, M. Puthenveedu, A. Henry, M. von Zastrow; Psychiatry, University of California San Francisco, San Francisco, CA

Membrane cargo is generally thought to be a passive player in the process of endocytic recycling. Contrary to this longstanding view, using live cell imaging of individual receptor recycling events, we show that the beta-2 adrenergic receptor (B2AR), actively regulates its own recycling. B2AR signaling, via activation of protein kinase A (PKA), significantly reduced the frequency of individual exocytic events mediating surface delivery of B2ARs from the Rab4-dependent recycling pathway. The PKA substrate essential for this regulation was identified as the B2AR itself, and mapped specifically to a single site in its carboxyl-terminal cytoplasmic domain. Strikingly, B2AR phosphorylation also regulated the recycling of the transferrin receptor, an independent cargo that is co-packaged in recycling vesicles with the B2AR. We conclude that B2AR phosphorylation provides a mechanism for cargo-mediated regulation over an entire recycling pathway, revealing a novel link between receptor signaling and membrane trafficking.

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Exocytosis of STI1 by Astrocytes Acts as a Prion Protein-Dependent Neurotrophic Factor.

C. A. Hartmann[1, 2], G. N. Hajj[1], M. Lopes[1], F. R. Lima[3], I. Porto-Carreiro[3], M. A. Prado[4], R. Linden[3], V. R. Martins[1]; [1]Molecular and Cellular Biology Laboratory, Ludwig Institute for Cancer Research, São Paulo, Brazil, [2]Chemistry Institute, University of São Paulo, São Paulo, Brazil, [3]Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [4]University of Western Ontario, Robarts Research Institute, London, ON, Canada

The physiological functions of cellular prion protein (PrPC) are under intense investigation, particularly those associated with brain development. In neurons, the association of PrPC with its ligand, the co-chaperone Stress Inducible Protein 1 (STI1) induces neuritogenesis and neuroprotection via ERK (Extracellular Regulated Kinase) and PKA (Protein Kinase A) signaling pathways, respectively. Herein, we demonstrated that STI1 can be secreted by astrocytes, even though it lacks a secretory signal sequence. This secretion is independent on the classical secretory pathway mediated by the Golgi apparatus, since Brefeldin A and Monensin had no blocking effect on it. The secreted STI1 is found in both a soluble form and in vesicles/aggregates that can be precipitated by ultracentrifugation. The evaluation of vesicles/aggregates purified from conditioned medium from wild-type and PrPC-null primary astrocytes by western blots demonstrates that STI1 is present in the fractions positive for transferrin receptor, HSP70, HSP90 and PrPC. However, STI1 secretion by exosomes was ruled out since very few of these vesicles were present in astrocytes`s conditioned medium evaluated by electron microscopy. Interesting, STI1 is found in vesicles of about 10-40 nm. GFP-STI1 expressed by transfected astrocytes is secreted in the same manner as endogenous STI1 and can be bound and internalized by neurons. The STI1 secreted by astrocytes activates the ERK1/2 signaling pathway in wild-type hippocampal neurons but not in PrPC-null neurons and anti-STI1 antibody blocks this activation. Remarkable, the protein content recovered from conditioned medium from PrPC null astrocytes is significantly lower than that from wild-type astrocytes. Taken together these findings indicate that STI1 is a neurotrophic factor secreted by astrocytes whose activity is dependent on the expression of PrPC at the neuronal surface. Moreover PrPC expression in astrocytes can modulate the secretion of vesicles or their protein content. Supported by FAPESP, CNPq and HHMI.

898/B97

Spatial and Kinetic Regulation of the Exocyst by PI(4, 5)P2 and Cdc42.

X. Zhang, B. He, K. Orlando, W. Guo; Department of Biology, U. Penn, Philadelphia, PA

The exocyst is an octameric protein complex implicated in tethering post-Golgi secretory vesicles at the plasma membrane in preparation for fusion. How the exocyst is targeted to and physically associates with specific domains of the plasma membrane, and how its functions at those regions are regulated are unclear. Here we report that the exocyst component Sec3 directly interacts with PI(4,5)P2 through its N-terminus; and this interaction is conserved in both yeast and mammalian cells. We have also identified key residues in Sec3 adjacent to the PI(4,5)P2-binding region that are critical for its binding to the GTP-bound form of Cdc42, a master regulator of cell polarity. Fluorescence Recovery after Photobleaching (FRAP) analysis indicates that the N-terminus of Sec3 is essential for its targeting to sites of polarized

secretion. Genetic analyses in yeast indicate that the interactions of Sec3 with PI(4,5)P2 and Cdc42 controls its function in exocytosis and cell polarity. Genetic screens using a Sec3 N-terminal truncation mutant have identified factors that spatially and kinetically regulate secretion. We propose that the interactions of Sec3 with phospholipids and Cdc42 play important roles in vesicle tethering during polarized exocytosis.

899/B98

Vacuolin-1-modulated Exocytosis and Cell Resealing Depend on Cell Origin and Mode of Activation.

P. Draber, G. M. Shaik, L. Draberova; Signal Transduction, Institute of Molecular Genetics, Prague 4, Czech Republic

Exocytosis and endocytosis are important cellular functions of outward and inward vesicular transport involved in intra- and inter-cellular communications. The role of lysosomal exocytosis and endocytosis in repair of damaged plasma membrane is incompletely understood. A small chemical vacuolin-1, which induces formation of large swollen structures derived from lysosomes and endosomes, was shown to inhibit Ca2+ ionophore-induced exocytosis depending on experimental conditions used. In human HeLa cells vacuolin-1 inhibited ionomycin-induced lysosome exocytosis but not repair of damaged plasma membrane, suggesting that lysosomes are dispensable for membrane resealing. Further studies performed under slightly different conditions however showed normal exocytosis and membrane resealing in vacuolin-1-pretreated HeLa and NRK cells, despite formation of the large swollen structures. These data suggested that lysosomes cannot be excluded as possible agents of membrane repair in vacuolin-1-treated cells. Because ionomycin is not physiological Ca2+ inducer and has some serious side effects, including a decline in ATP content of the cells, we decided to examine the effect of vacuolin-1 on exocytosis in specialized secretory cells activated by physiological and non-physiological activators. When rat basophilic leukemia (RBL) cells were activated through the high-affinity IgE receptor enhanced exocytosis was observed in vacuolin-1-pretreated, compared to activated control cells. Under identical conditions of activation, vacuolin-1 inhibited exocytosis in bone marrow-derived mast cells (BMMCs). Thapsigargin- and Ca2+ ionophore A23187-induced exocytosis also showed different sensitivity to inhibitory effect of vacuolin-1. If the cells were permeabilized with bacterial toxin streptolysin O, vacuolin-1 enhanced the resealing in RBL cells but inhibited it in BMMCs. Our data indicate that the inhibitory effect of vacuolin-1 on exocytosis and repair of injured plasma membrane depends not only on conditions of activation but also on cell origin. We propose that lysosomal exocytosis is involved in membrane repair even in professional secretory cells.

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Utilizing Multi-Angle TIRF Microscopy to Investigate Vesicle Exocytosis.

J. W. Goss, C. Fernandes, A. Karpikov, D. Toomre; Cell Biology, Yale University Medical School, New Haven, CT

The process of exocytosis is essential for a variety of cellular processes from regulation of cell size to release of hormones and neurotransmitters. However, the mechanism by which secretory vesicles become stationary at the plasma membrane (known as 'tethering') and then go on to fuse is unclear. Understanding the process of vesicle tethering and its regulation is of great importance. To address the question of vesicle tethering at the plasma membrane, we have implemented new instrumentation in the form of a multi-angle TIRFM, which provides superior axial resolution compared to commercial TIR microscopes. Using this system we have acquired multi-angle data of three different objects: large (20 micron) fluorescent beads, small point-source (<50nm) beads bound to cells, and cells with fluorescence-labeled microtubules. These samples provide gold standards for calibration. Our data with this multi-angle TIRFM indicates that we can achieve variable penetration (~100-500nm), and equally importantly we observe homogenous illumination and lack of interference fringing and coma-type artifacts that are prevalent in commercial TIRFM. This new technology provides us with a tool to explore factors that regulate the trafficking of vesicles as they approach the plasma membrane, and regulation of tethering and exocytosis.

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Abnormal Synaptic Vesicle Biogenesis and Neurotransmission in Dysbindin-Null Mice.

Y. Feng[1], X. Chen[2], X. Zhen[3], Z. Zhou[2], W. Li[1]; [1]Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China, [2]Institute of Molecular Medicine, Peking University, Beijing, China, [3]Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai, China

Synaptic vesicle (SV) shares common features with lysosome-related organelles, such as containing lysosome associated membrane proteins (LAMPs) and lower luminal pH. We hypothesize that the defect in dysbindin or BLOC-1 may affect the biogenesis of SVs. Here we have shown that the SVs in the adrenal chromaffin cells and the glutamatergic hippocampal neurons in sdy (dysbindin-null) mice were reduced in number and enlarged in size. By applying amperometry and whole-cell patch clamp in slices of sdy mice, we recorded slower quantal release, less release probability, and smaller readily releasable pool size. The steady-state of a synaptic release regulator, snapin, was reduced when dysbindin is null, which suggests dysbindin may be involved in regulating synaptic priming or fusion mediated by snapin. Furthermore, sdy exhibited schizophrenia-like behaviors showing cognitive deficits and social withdrawal, which may be resulted from the impaired transmission efficacy. Our results suggest that dysbindin plays a crucial role in regulating exocytosis and biogenesis of vesicles in hippocampal neurons, suggesting its possible involvement in the pathogenesis of schizophrenia.

902/B101

Regulated Exocytosis of Lysosomes in Polarized Epithelial Cells.

A. Lakkaraju[1,2], D. Gravotta[1,2], E. Rodriguez-Boulan[1,2,3]; [1]Dyson Vision Research Institute, Cornell University Weill Medical College, New York, NY, [2]Ophthalmology, Cornell University Weill Medical College, New York, NY, [3]Cell Biology, Cornell University Weill Medical College, New York, NY

Localized increase in intracellular calcium has been shown to induce fusion of conventional (non-secretory) lysosomes with the plasma membrane in non-polarized cells. Lysosome exocytosis provides material for plasma membrane repair and may also be a mechanism to get rid of cellular debris. Here, we investigated the polarity and molecular machinery involved in lysosome exocytosis in polarized epithelial cells. Polarized Madin-Darby canine kidney (MDCK) cells were treated with the calcium ionophore ionomycin (0-10 μM) for 10 minutes at

37C. The short exposure to ionomycin did not affect the tight junctions (assessed by ZO-1 immunostaining) or the trans-epithelial electrical resistance of the monolayer. Exocytosis of lysosomes was monitored by the appearance of the lysosome-associated membrane protein Lamp-2 on the plasma membrane and by the release of the lysosomal hydrolase β-hexosaminidase into the extracellular medium. MDCK cells showed a concentration-dependent increase in lysosomal exocytosis - at 5 μM, ionomycin induced the appearance of Lamp-2 exclusively on the basolateral surface in ~10-20% of cells, and this increased to >50% in response to 10 μM ionomycin. Treating cells with 5 μM ionomycin and the actin depolymerizing drug cytochalasin D increased the frequency of Lamp-2 externalization in agreement with previous reports that cortical actin is a barrier to exocytosis. Interestingly, cytochalasin D also altered the polarity of fusion and Lamp-2 was found on both the basolateral and apical membranes. Depolymerizing microtubules with nocodazole had no effect on lysosome exocytosis. We next investigated the role of the epithelial-specific clathrin adaptor AP1B in this process. MDCK cells lacking the μ1B subunit of the AP1B complex showed an amplified response to 5 μM ionomycin compared to wild-type cells. Lamp-2 was detected on the surface of ~80% of cells lacking μ1B, predominantly on the apical membrane. These data suggest that there is a hierarchy to lysosome exocytosis in polarized epithelial cells - fusion initially occurs at the basolateral surface and then at the apical membrane - and that the clathrin adaptor AP1B likely acts as a regulator of the extent and polarity of lysosome fusion.

903/B102

Plasticity in the v-SNARE Requirements for GLUT4 Trafficking in Adipocytes.

P. Zhao[1], L. Yang[2], J. A. Lopez[1], D. James[1], T. Xu[2]; [1]The Garvan Institute of Medical Research, Sydney, NSW, Australia, [2]National Laboratory of Biomacromolecules, Institute of Biophysics, Beijing, China

Vesicle transport in eukaryotic cells is regulated by SNAREs, which play an intimate role in regulating the specificity of vesicle fusion between discrete intracellular organelles. In mammalian cells several v-SNAREs have been described that form complexes with cell surface t-SNAREs and so it remains unclear if this demarcates a degree of plasticity within one single trafficking step or several discrete steps each regulated by a unique combination of SNAREs. In addition, while SNAREs have been shown to play an essential role in vesicle fusion it is unknown if they also play a role at a pre-fusion step in vivo. In the present study we have addressed both of these issues in the context of insulin regulated GLUT4 trafficking in adipocytes using a combination of knock out mice, clostridial toxins and TIRF microscopy to interrogate the function of VAMPs 2, 3 and 8. Our studies reveal that disruption of either VAMPs 2 or 8 alone, or VAMP2 & VAMP3 was without significant effect on insulin-regulated GLUT4 trafficking, whereas disruption of VAMPs 2, 3 and 8 together completely disrupted insulin stimulated GLUT4 translocation due to a block in vesicle docking with the plasma membrane as measured by high resolution TIRFM. These defects could be rescued with re-expression of VAMP2, VAMP3 or VAMP8. These data indicate a lack of specificity in the requirement for v-SNAREs in trafficking to the plasma membrane, and further define an important role for the v-SNARE both in docking and fusion.

904/B103

Live Imaging of Human Adipocytes: The Mystery of GLUT4 Trafficking.

K. G. Stenkula[1], V. A. Lizunov[2], S. W. Cushman[1], J. Zimmerberg[2]; [1]National Institute of Diabetes and Digestive and Kidney Diseases, NIH, Bethesda, MD, [2]National Institute of Child Health and Human Development, NIH, Bethesda, MD

Insulin promotes glucose uptake by recruiting glucose transporter-4 (GLUT4) from intracellular compartments to the plasma membrane, to facilitate diffusion of glucose into the cell. However, the mechanism regulating the insulin-induced trafficking and fusion of GLUT4 is still unknown. In this study we introduced a technique to simultaneously visualize GLUT4 and glucose uptake in live adipose cells isolated from human subjects. Red fluorescent-tagged GLUT4 was successfully expressed in human adipose cells with a transfection efficiency above 50%. GLUT4 was localized in both mobile vesicles and stationary clusters at the plasma membrane. Insulin stimulation reduced the number of mobile GLUT4 vesicles and induced GLUT4 accumulation in the stationary clusters. At a later stage (>20 min after stimulation), we found GLUT4 diffused into the plasma membrane. The uptake of glucose was monitored using a green fluorescent glucose analog (NBDG). Surprisingly, the insulin-stimulated NBDG uptake preceded the GLUT4 diffusion throughout the plasma membrane, suggesting that GLUT4 clusters were responsible for the initial increase in glucose uptake. We further tested GLUT4 association with Clathrin-coated pits and Caveolae, both of which have been implicated in the recycling of GLUT4. Mobile GLUT4 vesicles did not have significant colocalization with either Clathrin or Caveolin. However, some GLUT4 clusters showed transient recruitment of Clathrin that coincided with quantum removal of GLUT4 from the clusters, indicating single endocytic events. No clustering of GLUT4 was observed at pre-existing Clathrin patches. In summary, we suggest that in human adipose cells, insulin-regulated fusion of GLUT4 vesicles, glucose uptake, and subsequent endocytosis all take place at the identified clusters without significant redistribution of GLUT4 into the plasma membrane.

905/B104

The Exocyst Binds Munc18c to Regulate Basolateral t-SNARE Assembly and Polarized Trafficking in Epithelial Cells.

J. A. Lopez[2], K. S. Spiczka[1], D. James[2], C. Yeaman[1]; [1]Anatomy & Cell Biology, University of Iowa, Iowa City, IA, [2]Diabetes and Obesity Research Program, Garvan Institute of Medical Research, Sydney, NSW, Australia

The Exocyst is a multisubunit complex required for polarized membrane trafficking, but basic questions persist about how this essential complex functions during exocytosis. RNAi-mediated knockdown of the Sec6 subunit reduced trafficking efficiency of basolateral proteins (eg. E-cadherin, VSVG) in several epithelial cells (MDCK, Caco-2, HBECs). Surprisingly, ectopic expression of a truncated Sec6 protein consisting of just the carboxyl-terminal half (Sec6CT) was sufficient to restore basolateral trafficking. In contrast, Sec6NT, which binds other Exocyst subunits (eg. Sec5, Sec8), did not rescue the trafficking defect in Sec6-knockdown cells. To explore the mechanism involved, we sought to identify novel proteins that interact with Sec6CT. Yeast two-hybrid analysis and in vitro binding assays revealed that Sec6CT interacts with Munc18c, a regulator of the basolateral t-SNARE Syntaxin4 (STX4). Neither Sec6NT nor full-length Sec6 bound Munc18c, suggesting the possibility that either multimerization or conformational changes within Sec6 regulate Munc18c binding. Sec6CT binding required an amino-terminal domain of Munc18c previously shown to bind STX4. However, Sec6CT bound Munc18c in a manner that was neither competitive nor allosteric with STX4. Sec6 knockdown did not alter the localization of either STX4 or SNAP23 in epithelial cells; co-

immunostaining of the two proteins showed strong co-localization at the basolateral membrane. In contrast, Sec6 knockdown led to a complete dissociation of Munc18c from the basolateral membrane. Moreover, stable association of STX4 with SNAP23 was significantly affected by Sec6 reduction. Gel filtration analysis and co-immunoprecipitation studies showed very little association between these t-SNAREs in control cells, but that substantial levels of STX4:SNAP23 complex accumulated when Sec6 expression was reduced. Ectopic expression of Sec6CT in these cultures restored basal levels of STX4:SNAP23 complex. Collectively, these results provide new insights into the mechanism by which the Exocyst functions during vesicle tethering and docking and hint at novel regulatory events during polarized membrane trafficking.

906/B105

Changes in Inter-Subunit Interactions Accompany Exocyst Recruitment to Plasma Membrane and Correlate with its Activity.

K. S. Spiczka, N. J. Andersen, C. Yeaman; Anatomy & Cell Biology, University of Iowa, Iowa City, IA

Spatial regulation of exocytosis in cells relies on the Exocyst, an octameric protein complex believed to function in tethering vesicles to fusion sites at the plasma membrane. Nevertheless, our understanding of mechanisms regulating Exocyst assembly, localization and function are incomplete. Here, we have exploited a panel of anti-Sec6 monoclonal antibodies (mAbs) to probe Exocyst configurations in different compartments of epithelial MDCK cells. In these cells, the entire pool of Sec6 was present in a large complex with Sec3, -5, and -8, as shown by gel filtration and co-immunoprecipitation studies. We mapped epitopes recognized by ~25 distinct mAbs to one of four Sec6 segments. Surprisingly, mAbs that bound epitopes in each segment labeled distinct subcellular structures. In general, antibodies to epitopes in N-terminal domains labeled Sec6 in either cytosolic or endosomal pools, whereas those that bound epitopes in C-terminal domains labeled plasma membrane-associated Sec6. In this latter group, we identified antibodies that labeled distinct Sec6 populations at adherens junctions, desmosomes and vimentin-type intermediate filaments. That each antibody was specific was verified by both Sec6 RNAi and competition with fusion proteins containing each domain. To determine whether compartment-specific exposure of different epitopes correlated with differential stability of interactions between Sec6 and other Exocyst subunits, we quantified relative amounts of each subunit that co-immunoprecipitated with Sec6 when antibodies to N-terminal or C-terminal epitopes were used. Antibodies to Sec6NT co-precipitated more Sec5, -10, -15 and Exo84 than did those to Sec6CT. In contrast, antibodies to Sec6CT co-precipitated more Sec3 and Sec8 than did those to Sec6NT. These data are consistent with a model of Exocyst regulation in which molecular rearrangements occur within the octameric holocomplex that expose the C-terminal domain of Sec6 when the complex is plasma membrane-bound and conceal it when the complex is in the cytoplasm. Therefore, the Exocyst might behave as a “Rubik's Cube”, in which intersubunit interactions change when the complex is engaged in transport vesicle tethering and docking.

Membrane Receptors & Protein Trafficking

907/B106

Mis-Localisation and Cell Surface Rescue of Melanocortin-1 Receptor Variants.

K. A. Beaumont, R. A. Newton, P. J. Cassidy, J. Stow, R. A. Sturm; Institute for Molecular Bioscience, University of Queensland, Brisbane, QLD, Australia

The Melanocortin-1 Receptor (MC1R) is a seven transmembrane G-protein coupled receptor involved in the regulation of skin and hair pigmentation. The MC1R gene is highly polymorphic in Caucasian populations with a number of MC1R variant alleles associated with red hair, fair skin, poor tanning and skin cancer risk. We aimed to characterise the intracellular localisation and function of 9 common variant receptors in order to understand the molecular mechanisms underlying pigmentation phenotypes. Immunofluorescence and ligand binding studies demonstrated for the first time that the V60L, D84E, R151C, I155T, R160W and R163Q variant receptors had reduced cell surface expression compared to wild type MC1R. This resulted in a corresponding decrease in responses to MC1R agonist. Glycosylation studies indicated that the altered localisation of some MC1R variants is due to receptor mis-folding and ER retention, while other variant receptors may be retained in the Golgi complex. The R142H and D294H variants had relatively normal cell surface expression, however these receptors had defects in G-protein coupling. Interestingly the V92M variant, which previously appeared to be pseudo-wild type, was in fact localised to melanosomes. The melanosome is responsible for the synthesis and storage of the melanin pigment, the presence of V92M receptor here may have implications for the association of the V92M allele with the pigmentation phenotypes, or indicate a novel role for wild type MC1R at the melanosome. Preliminary studies using low temperature, chemical or pharmacological chaperones indicated that the cell surface expression of some MC1R variants could be rescued. This resulted in a restoration of signaling ability after stimulation with agonist. These studies have revealed for the first time that the most common mechanism of MC1R variant receptor dysfunction is mis-localisation or mis-trafficking of the receptor. The use of pharmacological chaperones holds promise for future drug based therapies that are able to rescue the cell surface expression and function of MC1R variants associated with skin cancer risk.

908/B107

Intracellular Trafficking of LRP9 is Dependent on Two Acidic Cluster/Dileucine Motifs.

R. Boucher, H. Larkin, J. Brodeur, C. Lavoie; Université de Sherbrooke, Sherbrooke, QC, Canada

LDL receptor-related protein 9 (LRP9) is a distant member of the low-density lipoprotein receptor (LDLR) superfamily. To date, there are no reports on the cellular distribution of LRP9 or the signals responsible for its localization. Here, we investigated the intracellular localization and trafficking of LRP9. Using confocal microscopy, we demonstrated that LRP9 was not present at the plasma membrane but co-localized with various markers of the trans-Golgi network (TGN) and endosomes. This co-localization was dependent on the presence of two acidic cluster/dileucine (DXXLL) motifs in the cytoplasmic tail of LRP9, which interact with GGA proteins, clathrin adaptors involved in transport between the TGN and endosomes. LRP9 is the first example of a transmembrane protein with an internal GGA-binding sequence in addition to the usual C-terminal motif. An inactivating mutation (LL --> AA) in both DXXLL motifs, which completely inhibited the interaction of LRP9 with GGA proteins, led to an intracellular redistribution of LRP9 from the TGN to early endosomes and the cell surface, indicating that

the two DXXLL motifs are essential sorting determinants of LRP9. In conclusion, our results suggest that LRP9 cycles between the TGN, endosomes and the plasma membrane through a GGA dependent-trafficking mechanism.

909/B108

Src Homology 3 Domain Containing, Ysc84-Like 1 (SH3YL1) Act as Dominant Negative Molecule for Noxa1.

Y. Yu, H. Jung, Y. Bae; Division of Life and Pharmaceutical Sciences, Ewha womans university, Seoul, South Korea

It has been well established that NADPH oxidase 1 (Nox1) requires two cytosolic proteins, Nox organizer 1 (Noxo1) and Nox activator 1 (Noxa1) and GTP-binding protein Rac1. In this report, we show that a novel protein, SH3YL1 (Src homology 3 domain containing, Ysc84-like 1) can interact with NADPH oxidase complex and regulate their activity. GST pulldown and co-immunoprecipitation experiments indicate that SH3YL1 interacts with Noxo1. Membrane translocation of SH3YL1 with Noxo-GFP was increased in response to EGF stimulation. We analyzed the function of SH3YL1 in NADPH oxidase 1 activity. Expression of SH3YL1 into HEK293 cells expressing Nox1/Noxo1/Noxa1 resulted in decreased ROS generation in response to EGF. Moreover, interaction of Noxo1 with Noxa1 was disrupted in HEK293 cells expressing SH3YL1. These results suggest that SH3YL1 acts as dominant negative molecule in NADPH oxidase complex formation.

910/B109

Selective Utilization of Lipid Rafts during FcγRIIA Triggered Endocytosis and Phagocytosis.

J. A. Vieth[1], M. Kim[2], X. Pan[2], A. D. Schreiber[2], R. G. Worth[1]; [1]Department of Medical Microbiology & Immunology, University of Toledo College of Medicine, Toledo, OH, [2]Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA

Immunoglobulin G (IgG) dependent effector activity is an important contributor to cell-mediated host defense against pathogens, as well as the dysfunction associated with such autoimmune diseases as systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA). Various cell types including macrophages and neutrophils participate in pathogen destruction and tissue damage by expressing a family of Fcγ receptors (FcγR) which recognize monomeric as well as complexed IgG. One member of this family, FcγRIIA, is a transmembrane glycoprotein previously shown to mediate binding and internalization of large IgG-coated targets (phagocytosis) and small IgG-containing complexes (IC) (endocytosis). FcγRIIA-mediated phagocytosis and endocytosis differ in their requirement for actin versus clathrin as well as varied dependency on specific kinases and phosphatases. FcγRIIA has also been observed to migrate into lipid rafts (detergent insoluble, cholesterol and sphingolipid containing membrane domains) upon ligation. Employing FcγRIIA transfected CHO cells, we have shown that the amount of raft-associated FcγRIIA more than doubles in response to binding of IgG coated sheep erythrocytes (EA) while binding of smaller targets (IC) results in less lipid raft association than EA. The objective of this study is to identify the functional consequence(s) of this discrepancy in lipid raft localization. Disruption of lipid rafts with 5mM methyl-β-cyclodextrin (MβCD) nearly abolishes binding of large IgG coated targets (85% inhibited) while binding of IC is only minimally dependent on lipid rafts (38% inhibition at 5mM MβCD). Additionally, disruption of lipid rafts with 5mM MβCD after binding inhibited phagocytosis by almost 65% while endocytosis was only modestly affected (24% inhibition at 5mM MβCD). Our current observations suggest that lipid raft utilization may play a crucial role in the differences between phagocytosis and endocytosis and that these differences may arise early in the process of ligand recognition.

911/B110

Nuclear Translocation of β-Dystroglycan Reveals a Distinctive Trafficking Pattern of Autoproteolyzed Mucins.

M. Oppizzi[1], A. Akhavan[1], M. Singh[1], J. E. Fata[2], J. L. Muschler[1]; [1]Research Institute, California Pacific Medical Center, San Francisco, CA, [2]Department of Biology, College of Staten Island, Staten Island, NY

Dystroglycan (DG) is a cell surface extracellular matrix receptor, altered functions of which are implicated in muscular dystrophies and cancers. DG belongs to the membrane-tethered mucin family and is composed of an extracellular (alpha-DG) and transmembrane (beta-DG) subunits stably coupled at the cell surface. These two subunits are generated by autoproteolysis of a precursor within a distinctive protein motif called an SEA (sea urchin-enterokinase-agrin) domain, however, the purpose of this cleavage is uncertain. Here we demonstrate that beta-DG exhibits diversified compartmentalization, free of the alpha-DG subunit, localizing to cytoplasm and nucleoplasm in addition to the plasma membrane. The full-length and glycosylated beta-DG subunit translocates to the nucleus in a process that occurs independent of alpha-DG ligand binding. This phenomenon is, thus far, indistinguishable from translocation of the C-terminal subunit of the related MUC1 oncoprotein, also a dimeric membrane-tethered mucin created by SEA autoproteolysis. Additionally, we reveal that beta-DG is directed to the nucleus by an intrinsic nuclear localization signal. These findings point to novel intracellular functions for beta-DG, with possible disease implications, and suggest that SEA autoproteolysis serves to enable independent functions of mucin transmembrane subunits, enacted by segregated subcellular compartmentalization.

912/B111

Study of the Endocytic Pathway of ITIM-Bearing Inhibitory Receptors: CD94/NKG2A and LAIR-1.

G. Peruzzi[1], M. Masilamani[2], S. Narayanan[1], F. Borrego[1], J. E. Coligan[1]; [1]NIAID/National Institutes of Health (NIH), Rockville, MD, [2]Division of Pediatric Allergy & Immunology, Department of Pediatrics, Anbg 17-46A, Mount Sinai School of Medicine, New York, NY

Natural killer (NK) cells express a variety of activating receptors that are prevented from inappropriately activating cells by co-expressed inhibitory receptors. Our group has recently characterized the trafficking pattern of the ITIM-containing inhibitory receptor CD94/NKG2A, expressed by NK and T cells, that recognizes HLA-E ligand expressed by most normal cells. Ligation often induces receptor downregulation. In the case of CD94/NKG2A, this could potentiate activation receptor(s) induced responses to normal bystander cells. Our previous studies showed that CD94/NKG2A is long-lived and continuously recycles to the cell surface, and that interaction with ligand does not lead to down-regulation. The abbreviated trafficking pattern of CD94/NKG2A, perhaps to insure surface expression, was found to occur by an amiloride-sensitive, Rac1-dependent macropinocytic-like process; however, it does not require clathrin, dynamin, ADP ribosylation factor-6, phosphoinositide-3 kinase or the actin cytoskeleton. Once endocytosed, CD94/NKG2A traffics to early endosomal antigen 1+, Rab5+ early endosomes. It does appear in Rab4+ early/sorting endosomes, but, in the time period examined, fails to reach Rab11+ recycling or Rab7+ late

endosomes, or lysosome-associated membrane protein-1+ (Lamp-1) lysosomes. The leukocyte associated inhibitory receptor (LAIR)-1 is another ITIM containing inhibitory receptor expressed by all leukocytes whose ligand are collagens. The work in progress is aimed at determining if the unique endocytic mechanism described for CD94/NKG2A is similar for LAIR-1, i. e. is there a common bioprocessing theme related to the function and/or structure of ITIM-bearing receptors.

913/B112

CD2-CD58 Interaction Organizes Membrane Microdomains and Facilitates T cell Receptor Signaling In Jurkat T Cells.

Y. Kaizuka, A. Douglass, R. Vale; HHMI and Department of Cellular and Molecular Pharmacology, University of California San Francisco, San Francisco, CA

The interaction between a T cell and an antigen-presenting cell (APC) can trigger a signaling response that leads to T cell activation. In addition to the well-studied T cell receptor (TCR) signaling cascade, various "co-receptors" also contribute to T cell activation, but their roles, biochemical mechanisms, as well as coordination with TCR are still poorly understood. Here, using a model system consisting of Jurkat T cells interacting with a planar lipid bilayer (a simplified activation system that mimics the APC), we show that CD58 incorporated into planar lipid bilayers stimulates microdomain co-clustering of CD2 and several signaling molecules (including the TCR z chain, Lck, and LAT) and leads to down stream signaling events that resemble the pathway initiated by TCR activation. We also show that CD58-CD2, in combination with TCR activation, produces a greater signaling response than can be obtained by activating the TCR alone. We propose that the CD58-CD2 interaction orchestrates a downstream signaling system that promotes T cell activation and that this activation process involves a dramatic spatial reorganization of signaling molecules in the plasma membrane in a way that is similar to but also distinct from that described for the TCR.

914/B113

The Fc Receptor Complex Captured from the Surface of Live RAW Macrophages and Sequenced by LC-ESI-MS/MS.

V. Barbisan, J. Marshall; Ryerson University, Toronto, ON, Canada

The Fc receptor is expressed on the surface of RAW macrophages that can engulf IgG opsonized particles by phagocytosis. Cell surface receptors found on the plasma membrane are key targets for modulating cellular responses. However, isolation and characterization of these receptors and the identification of proteins involved is challenging. Here, the Fc receptor complex has been captured from the surface of live cells with the use of microbeads coated with the receptors ligand, alongside a number of controls, and analyzed by LC-MS/MS. Live-cell Affinity Receptor Chromatography (LARC) [Analytical Biochemistry 380: 235-248] resulted in the identification of approximately 250 proteins which are found to be specifically associated with the activated Fc receptor in RAW 264.7 macrophage cells. TIC chromatograms of the crude alone, crude and IgG coated beads and crude with BSA appeared different, indicating the identification of different proteins. The proteins isolated were analyzed for protein-protein interactions using various analytical techniques including data mining through the use of iHOP, protein interaction databases such as Cytoscape, or Osprey and theoretical literature searches, in order to create a signaling interaction pathway involving the proteins that are activated and associated with the Fc receptor during receptor-mediated phagocytosis. Proteins that had 3 unique peptide sequences, a cumulative score of at least 2400 and also had at least 3 protein-protein interactions were assembled into a model of the macrophage Fc receptor. The Fc receptor model was found to be in close agreement with the previous genetic and biochemical literature and included many novel members of the regulatory network of proteins associated with the Fc receptor. Hence it appears it is possible to capture and identify the members of a cell surface receptor complex using live cell receptor chromatography.

915/B114

The Fc Receptor Complex Captured from the Surface of Live Human Neutrophils, Sequenced by LC-ESI-MS/MS.

V. Petrenko, J. Marshal; Chemistry and Biology, Ryerson University, Troronto, ON, Canada

Phagocytosis is a complex and dynamic process of particle engulfment and is an important mediator of innate and adoptive immune defenses. The Fc receptor complex is a well understood receptor at the genetic and cellular levels. Although many signaling events during phagocytosis have been described, much is still unclear including many proteins and their isoforms involved. The Fc receptor was captured from the surface of live human neutrophils using its cognate ligand affixed to microbeads. The cells were ruptured by French press and the beads collected though sucrose centrifugation. The complex was eluted from the beads in salts and then digested with trypsin and the residual hydrophobic proteins digested in buffered organic solvents. The peptides were identified using LC-ESI-MS/MS. The MS/MS data was screened for proteins that have three unique peptide sequences and a uniscore at the 5% confidence or greater which was the basis of acceptance of proteins into the mass spectra data. Crude extracts incubated with microbeads alone or blocked with IgG or BSA served as controls to eliminate false positives from non-specific binding. Identified proteins were then searched in iHOP for at least three different previous physical or regulatory associations or interactions between the identified proteins to create a model of the Fc receptor. Models were created with iHOP and exported into OSPREY where the model was visualized. Close to a 1000 proteins were specifically identified from the ligand coated beads bound to live cells. Some three hundred of these had at least three previously documented interactions with other members of the data set. Hence a model of FcγR mediated phagocytosis in human neutrophils has been constructed form LC MS/MS data obtained through LARC of IgG opsinized particles.

916/B115

ER-associated Degradation Pathway Is Involved in Nuclear Trafficking of ErbB-2.

L. Li[1], H. Chu[1], H. Sun[2], M. Hung[2]; [1]China Medical University and Hospital, Taichung, Taiwan, [2]The University of Texas M.D. Anderson Cancer Center, Houston, TX

We have demonstrated that ErbB-2 translocates into the nucleus through importin b1-mediated nuclear import by endosomal sorting, and transactivates transcription of cyclooxygenase-2 which has been known to be involved in tumor progression and metastasis. However, how the membrane-bound ErbB-2 can be released from the lipid bilayer and pass through the nuclear pore complex to move to the nucleus is still yet completely understood. Here, we report that ErbB-2 localizes to ER and interacts with Sec61a, a subunit of Sec61 channel, which is

known to mediate retrotranslocation of proteins in the ER to the cytosol (called ER-associated degradation pathway). Knocking down Sec61a using small interfering RNA oligonucleotides inhibits retrotranslocation of ErbB-2 to the cytoplasm and causes a nuclear transport defect of ErbB-2. However, ErbB-2 does not associate with a-mannosidase I which recognizes and removes mannose from the misfolded proteins, leading to protein extraction into the cytosol via sec61 channel. Knockdown of a-mannosidase I using siRNA increases retrotranslocation of ErbB-2 to the cytosol and to the nucleus, implying ErbB-2 may compete with misfolded proteins to bind Sec61a. These results suggest that part of ER-associated degradation pathway is likely involved in nuclear trafficking of ErbB-2 (supported by National Health Research Institutes NHRI-EX96-9603BC, NHRI-EX97-9603BC and National Science Council NSC95-2311-B-039-002).

Endocytic Machinery: Structure, Function, and Regulation (917 – 939)

917/B116

Regulation of Flotillin-Dependent Endocytosis by Fyn Kinase.

K. Riento, M. Frick, I. Schafer, B. Nichols; MRC-LMB, Cambridge, United Kingdom

Flotillin1 and flotillin2 co-assemble into plasma membrane microdomains that are involved in the endocytosis of molecules such as glycosyl phosphatidylinositol (GPI)-linked proteins [1-3]. Previous studies suggest that budding of flotillin microdomains from the plasma membrane is a tightly regulated process [4]. Here we demonstrate that endocytosis of flotillins is regulated by the Src family kinase Fyn. The Src kinase inhibitor PP2 prevents EGF-induced flotillin internalization [5]. Expression of active Fyn, but not Src or Lyn, causes redistribution of flotillin microdomains from the plasma membrane to intracellular organelles, while it has no effect on caveolin distribution or clathrin-dependent endocytosis. Using two partial Fyn constructs that form a functional kinase upon addition of rapamycin to cells, we show that flotillin internalization from the plasma membrane occurs shortly after Fyn activation. Fyn co-localizes and is co-internalized with flotillin microdomains. Phenylalanine mutations of tyrosine 160 on flotillin1 and tyrosine 163 on flotillin2, which are directly phosphorylated by Fyn, prevent Fyn-induced flotillin internalization. Uptake of the GPI-linked protein CD59 in flotillin-containing endocytic carriers is blocked by expression of the phenylalanine-mutated flotillin constructs. These data establish uptake of flotillin microdomains as a tyrosine kinase regulated endocytic process [6]. **References 1.** Glebov, O.O., Bright, N.A., and Nichols, B.J. (2006). Nat Cell Biol 8, 46-54. **2.** Payne, C.K., Jones, S.A., Chen, C., and Zhuang, X. (2007). Traffic 8, 389-401. **3.** Langhorst, M.F., Reuter, A., Jaeger, F.A., Wippich, F.M., Luxenhofer, G., Plattner, H., and Stuermer, C.A. (2008). Eur J Cell Biol 87, 211-226. **4.** Frick, M., Bright, N.A., Riento, K., Bray, A., Merrified, C., and Nichols, B.J. (2007). Curr Biol 17, 1151-1156. **5.** Neumann-Giesen, C., Fernow, I., Amaddii, M., and Tikkanen, R. (2007). J Cell Sci 120, 395-406. **6.** Liberali, P., Ramo, P., and Pelkmans, L. (2008). Annu Rev Cell Dev Biol 3, 3.

918/B117

The DHR-1 Domain of DOCK180 Binds to SNX5 and Regulates Cation-independent Mannose 6-phosphatate Receptor Transport.

S. Hara[1,2], E. Kiyokawa[1], S. Iemura[3], T. Natsume[3], T. Wassmer[4], P. Cullen[4], H. Hiai[5], M. Matsuda[1,2]; [1]Department of Pathology and Biology of Diseases, Kyoto University, Kyoto City, Japan, [2]Department of Signal Transduction, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan, [3]Biological Information Research Center, National Institute of Advanced Industrial Science and Technology, Tokyo, Japan, [4]Department of Biochemistry, University of Bristol, Bristol, United Kingdom, [5]Shiga Medical Center Research Institute, Shiga, Japan

DOCK180 is one of the two major binding partners of Crk oncogene product, and is the archetype of the DOCK180-family guanine nucleotide exchange factor for small GTPases Rac1 and Cdc42. DOCK180 has two conserved domains called DOCK Homology Region (DHR)-1 and -2, of which functions are to bind to phosphoinositides, and to activate Rac1 for cell invasion, respectively. To better understand the DHR-1 function, we searched for its binding proteins by direct nano-flow liquid chromatography/tandem mass spectrometry, and identified sorting nexin (SNX) 1, 2, 5, and 6, which comprise a multimeric protein complex mediating endosome-to-trans-Golgi-network retrograde transport of the cation-independent mannose 6-phosphate receptor (CI-MPR). We found that DOCK180 bound to SNX5 most efficiently, and localized at endosomes with SNX5. DOCK180 and SNX5, but not Rac1, depletion by RNA interference perturbed CI-MPR trafficking. Re-expression of the wild-type, but not the mutant DHR-1 of DOCK180 defective for binding to phosphoinositides and SNX5, was sufficient to restore CI-MPR trafficking. These data suggest that DOCK180 regulates retrograde transport of CI-MPR via SNX5 and lipids, and that this function is independent of its GEF activity toward Rac1.

919/B118

Morphology of Clathrin Lattices in Clathrin Light Chain-Depleted Cells.

P. N. Dannhauser, E. J. Ungewickell; Cell Biology, Hannover Medical School, Hannover, Germany

Clathrin light chains (CLC) are known to (i) stabilize clathrin triskelia, (ii) to regulate clathrin assembly in vitro and (iii) to link via their interaction with Hip1R clathrin coated vesicle dynamics to that of the actin cytoskeleton. Downregulation of the expression of CLC by RNAi was recently shown to re-arrange actin and in particular to affect the distribution of the AP-1 adaptor and that of the mannose-6-phosphate receptor (Poupon et al. 2007, PNAS 105, 168-173). Here we knocked down CLC in Hela cells and observed the cells for up to 6 days. Western blot analysis showed that 72 hrs after transfection the CLC were reduced by more than 80% and the clathrin heavy chain (CHC) by 20% - 30%. On subsequent days the amount of heavy chain continued to drop. The reduction of the cellular heavy chain concentration is consistent with previous notions that CLC stabilize the CHC. Immunofluorescence analysis performed on the third day after transfection showed that the CLC-depleted CHC shifts from internal membranes to the plasma membrane where it concentrates in large patches. This phenotype was rescued by microinjection of Alexa Fluor® 488-labeled heavy chains (488-CHC) complexed to CLC. In contrast microinjection of 488-CHC alone did not reduce the size of the patches. To explore the nature of the CHC-patches on the plasma membrane in more detail we examined cellular cortices of CLC-depleted HeLa cells by electron microscopy. We noted that the ventral plasma membrane was covered with unusual large and predominantly flat clathrin lattices. The ratio of flat to curved membranes was about 2:1. In

contrast the lattices in control cells were smaller and they contained numerous buds (ratio of flat to curved membranes ca. 1:2). We conclude that CLC influence directly or indirectly budding of clathrin coated vesicles.

920/B119

Rabex-5, a Guanine Exchange Factor for Rab5, Regulates the Ubiquitin-dependent Endocytic Trafficking of Neural Cell Adhesion Molecule L1.

Y. Aikawa[1], Y. Shigemi[1], M. Watanabe[1], V. Lemmon[2], K. Itoh[1]; [1]Laboratory of Molecular and Cellular Neurosciences, Tokushima Bunri University, Sanuki City, Japan, [2]The Miami Project to Cure Paralysis, University of Miami School of Medicine, Miami, FL

Dynamic turnover of a neural cell adhesion molecule L1 to and from the cell surface is central to neurite outgrowth and cell motility. Here we found that lys-63 linked ubiquitination of L1 on the cell surface is required for the maximal internalization and the sorting into lysosomes to degrade in Neuro-2a cells upon L1 antibody cross-linking which mimics L1-L1 homophilic interaction. During this ubiquitin-dependent endocytic trafficking, Rabex-5, a guanine exchange factor (GEF) for Rab5, specifically associates with the ubiquitinated L1 (Ub-L1) by way of the inverted ubiquitin-interacting motif (IUIM) at the N-terminal side, then it leads to facilitate the GEF activity of VPS9 domain at the C-terminal side. In contrast, Rabex-5A58D which is not able to associate with the Ub-L1 does not results in activation of the GEF activity, indicating that the association of the IUIM to the Ub-L1 on the cell surface induces the GEF activation of VPS9 domain for endocytic trafficking. RNAi knock-down of Rabex-5 specifically impairs the internalization of the Ub-L1 but not Ub-deficient mutant 11KR from the cell surface upon L1 antibody cross-linking. Co-expression of Rabex-5 with GDP-locked Rab5S34N, remarkably down-regulates the cell surface expression of L1 and accumulates the Ub-L1 on Rabex-5-induced vacuole, while co-expression of GTP-locked Rab5Q79L and/or the expression of Rabex-5N413A that Vps9 domain is constitutively activated restore this accumulation of the Ub-L1 on the vacuole. This suggests that the GEF activity of Vps9 domain depending on the nucleotide-bound form of Rab5 changes the binding affinity for Ub-L1. Overall, these results provide mechanistic insight into how cell surface pool of L1 upon L1-L1 homophilic interaction is spatially and temporally regulated by Rabex-5 activation, which in turn drives the removal of Ub-L1 from the cell surface.

921/B120

Comparative Genetic, Cell Biological and Developmental Analysis of Metazoan ESCRT Function By Efficient Isolation of Drosophila ESCRT-I, -II, -III Mutants.

T. Vaccari[1], T. Rusten[2], L. Menut[1], A. Brech[2], G. Wu[1], H. Stenmark[2], D. Bilder[1]; [1]UC berkeley, Berkeley, CA, [2]Centre for Cancer Biomedicine, University of Oslo, oslo, Norway

ESCRT proteins were initially isolated in yeast as a single functional set of conserved components controlling critical aspects of endosomal cargo sorting and multivesicular bodies (MVBs) biogenesis. Recent work has suggested that metazoan ESCRT proteins might play more functionally diverse roles and be in part dispensable for MVB biogenesis. However, the characterization of few null ESCRT mutants has so far hampered a thorough analysis of ESCRT functions in metazoans. We used a highly efficient genetic screening strategy to isolate null mutations in nearly half of the Drosophila ESCRT genes, including components of all three ESCRT-I, -II, -III complexes. All these ESCRT genes behave as neoplastic tumor suppressor genes and regulate signal transduction by controlling degradation of ubiquitinated molecules including signaling receptors Notch and EGFR in disc epithelia. In contrast, although components of all three complexes are required for plasma membrane stability in the germline, only ESCRT-II components control a previously reported oocyte polarity function. Finally, ultrastructural analysis of oocytes mutants for all the identified ESCRT components showed impaired MVB formation and aberrant yolk granule formation. Our work highlights the multiple cell biological and developmental roles of ESCRTs in Drosophila and provides the basis for extensive analysis of metazoan ESCRT function.

922/B121

The Association of Clathrin with Cellular Organelles Demonstrated by Quantum Dots Immuno-Electron Microscopy.

Y. Ogunkoya[2], B. M. Nickel[1], V. L. Gay[1], S. Murray[1]; [1]Dept Cell Biol/Physiol, Univ Pittsburgh Sch Med, Pittsburgh, PA, [2]Department of Biology, Southern University and Agricultural & Mechanical College, Baton Rouge, LA

An extensive body of research has been used to establish the importance of clathrin in the process of endocytosis. Clathrin-dependent endocytosis occurs at specialized anatomical structures called coated pits which detach from the plasma membrane to form cytoplasmic clathrin coated vesicles. With electron microscopy a bristle coat, composed of clathrin, has been demonstrated to surround coated pits and vesicles, but there is a need to evaluate the composition of the bristle coat surrounding other cellular organelles. In this study, quantum dot immuno-electron microscopic techniques were used to analyze the association of clathrin and one of its adaptor proteins, AP-2, with cytoplasmic structures in a human adrenal cortical cell line. Clathrin was demonstrated to associate with the plasma membrane and a variety of vesicle types, including the classic clathrin coated vesicles, pentilaminar annular gap junction vesicles, multivesicular bodies, and other unidentified structures. In addition, clathrin associated with connexin 43 (Cx43) gap junction plaques, where it preferentially localized to the cytoplasmic surface of the gap junction plaque membrane of one of two contacting cells. The adaptor protein, AP-2 was associated with the plasma membrane, coated pits, and with vesicles near the cell membrane. The electron micrographs obtained with the quantum dot technology allows an accurate and specific localization of clathrin and its adaptor protein with cellular organelles. Supported by NSF grant # MCB-0647748

923/B122

Regulation of Phosphatidylinositol 3, 5 Bis Phosphate, a Lipid with Unexpected Links to Neurodegenerative Disease.

N. Jin[1], C. Y. Chow[2], S. N. Zolov[1], J. L. Petersen[1], Y. Zhang[1], S. Park[1], J. E. Duex[1], M. H. Meisler[2], L. S. Weisman[1]; [1]Cell and Developmental Biology and The Life Sciences Institute, University of Michigan, Ann Arbor, MI, [2]Department of Human Genetics, University of Michigan, Ann Arbor, MI

The signaling lipid PI(3,5)P2 is generated on endosomes and regulates retrograde traffic to the trans-Golgi network. Physiological signals result in rapid, transient changes in PI(3,5)P2 levels. Mutations that lower PI(3,5)P2 cause neurodegeneration in mouse models and human

patients. While some of the proteins that control PI(3,5)P2 levels have been identified, the functions of a subset of the proteins were unknown. Here we report a bioinformatics analysis that predicts that Vac14, a protein required to control PI(3,5)P2 levels, is composed entirely of HEAT repeats and could function as a scaffold. Indeed, using yeast two-hybrid, co-immunoprecipitation, and sedimentation studies, we demonstrate that the PI(3,5)P2 regulatory proteins form a complex nucleated by Vac14. The lipid kinase Fab1 and the lipid phosphatase Fig4 bind Vac14 to form a core complex. The association with the complex of two regulators, Vac7 and Atg18, is required for the acute regulation of PI(3,5)P2. Moreover, we report that the mouse mutant ingls (infantile gliosis) results from a missense mutation in Vac14. Similar to the Vac14 and Fig4 null mouse mutants, the ingls mutant dies prematurely, and exhibits significant neurodegeneration. We show that the Vac14-ingls mutation has a partial defect and blocks the association of Vac14 with Fab1. The loss of interaction abolishes the ability of Vac14-ingls cells to rapidly and transiently raise PI(3,5)P2 levels in response to stimuli, and moreover lowers basal levels of PI(3,5)P2 approximately two-fold. This work elucidates the identity and interactions between protein components of the PI(3,5)P2 regulatory complex, and demonstrates that formation of the complex is essential for modulation of PI(3,5)P2 levels and for neuronal function.

924/B123

Structure of an Arrestin2/Clathrin Complex Reveals a Novel Site of Interaction That Modulates Receptor Trafficking.

D. Kang[1], R. C. Kern[1], M. Puthenveedu[2], M. von Zastrow[2], J. C. Williams[1], J. L. Benovic[1]; [1]Biochemistry & Molecular Biology, Thomas Jefferson University, Philadelphia, PA, [2]Cell & Molecular Pharmacology, University of California at San Francisco, San Francisco, CA

Non-visual arrestins play a pivotal role as adaptor proteins in regulating the signaling and trafficking of multiple classes of receptors. While arrestin interaction with clathrin, AP-2, and phosphoinositides contributes to receptor trafficking, little is known about the configuration and dynamics of these interactions. Here, we demonstrate that the X-ray crystal structure of an arrestin2/clathrin complex reveals two binding interfaces between these proteins. The intrinsically disordered clathrin binding box of arrestin2 interacts with a groove between blades 1 and 2 in the clathrin β-propeller domain while an 8 amino acid splice loop found solely in the long isoform of arrestin2 (arrestin2L) interacts with a binding pocket formed by blades 4 and 5 in clathrin. The apposition of the two binding sites suggests that they are exclusive and may function in higher order macromolecular structures. Biochemical analysis demonstrates direct binding of the splice loop in arrestin2L to clathrin while functional analysis reveals that both binding domains contribute to the receptor-dependent redistribution of arrestin2L to clathrin-coated pits. Taken together, these data provide a framework for understanding the dynamic interactions between arrestin2 and clathrin and reveal an essential role for this interaction in arrestin-mediated endocytosis.

925/B124

Functional Determinants of Ras Interference 1 Mutants Required for their Inhbitory Activity on Endocytosis.

A. Galvis, V. Balmaceda, H. Giambini, A. Conde, Z. Villasana, A. Barbieri; Department of Biological Sciences, Florida International University, Miami, FL

In this study, we initiated experiments to address the structural-function relationship of Rin1. A total of ten substitute mutations were created, and their effects on Rin1 function were examined. Of the ten mutants, four of them (P541A, E574A, Y577F, T580A) were defective in Rab5 binding, while three other Rin1 mutants (D537A, Y561F, T572A) partially interacted with Rab5. Biochemical studies showed that six of them (D537A, P541A, Y561F, E574A, Y577F, T580A) were unable to activate Rab5 in an in vitro assay. In addition, Rin1:D537A and Rin1:Y561F mutants markedly inhibited the activation of Rab5 upon ligand stimulation in intact cells. Consistent with the biochemical studies, we observed that these six Rin1 mutants have lost their ability to stimulate the endocytosis of EGF or form enlarged Rab5-positive endosomes. Mutantions in several other residues (Y506F, Y523F, Y578F) resulted in partial loss of Rab5 function. Based on these data, our results showed that mutations in the Vps9 domain of Rin1 lead to a loss-of-function phenotype, indicating a specific structure-function relationship between Rab5 and Rin1.

926/B125

A Separate Role for Clathrin Heavy and Light Chain Subunits in Cell Migration.

C. Chen[1,2,3], J. Bennetts[1,2,3], C. Esk[1,2,3], F. M. Brodsky[1,2,3]; [1]Biopharmaceutical Sciences, Pharmaceutical Chemistry, UCSF, San Francisco, CA, [2]Microbiology and Immunology, UCSF, San Francisco, CA, [3]Hooper Foundation, UCSF, San Francisco, CA

The clathrin triskelion is composed of heavy and light chain subunits and assembles into a polyhedral coat for membrane vesicles that mediate transport for endocytosis and organelle biogenesis. We assessed whether different roles in clathrin function can be assigned to the heavy and light chain subunits, using RNA interference of their expression and rescue of expression with siRNA-resistant constructs encoding wild-type and mutant light chain subunits. In HeLa cells, depletion of clathrin heavy chain (CHC) caused defects in transferrin uptake and altered the distribution of EEA1, LAMP1, AP1 and TGN46. Depletion of clathrin light chain (CLC) caused a mild version of these phenotypes, which was accounted for by reduction in CHC stability upon loss of CLC. Ectopic over-expression of CLC did not rescue these phenotypes, assigning CHC a key role in clathrin-mediated membrane traffic. Looking for pathways in which a phenotype for CLC would be more obvious, we tested effects of heavy and light chain depletion on cytoskeleton morphology and cell migration because of the known interaction between CLC and the actin-binding Hip proteins. Different actin phenotypes were caused by depletion of each clathrin subunit. In the Boyden chamber migration assay, depletion of each clathrin subunit had opposite effects, with CHC depletion enhancing migration and CLC depletion causing inhibition of migration. The reduced migration caused by CLC depletion was rescued by CLC but not by CLC with a mutation in the Hip protein binding site. These results suggest that CLC links clathrin with actin dynamics during cell migration via an interaction with Hip proteins. As a whole, these results highlight two roles for clathrin, one as a coat protein and the other as a scaffold for organizing actin via CLC function.

927/B126

Regulation of Clathrin Adaptor Function in Endocytosis: A Novel Role for the SAM Domain.

S. Di Pietro[1], D. Cascio[2], J. U. Bowie[2], G. Payne[3]; [1]Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO,

[2]Molecular Biology Institute, UCLA, School of Medicine, Los Angeles, CA, [3]Biological Chemistry, UCLA, School of Medicine, Los Angeles, CA

Adaptor proteins play important roles in endocytosis such as recognition of internalization signals in transmembrane cargo and binding clathrin. Sla1 serves as the adaptor for uptake of transmembrane proteins containing the NPFxD internalization signal, and is essential for normal functioning of the actin cytoskeleton during endocytosis. Sla1 link to clathrin is thought to be indirect, through interactions with other components of the endocytic clathrin coat. We have now found that Sla1 contains a clathrin binding sequence within an unstructured region of its primary structure. At variance with previously described clathrin box sequences (LΦXΦ[DE], where Φ is a bulky hydrophobic residue), the Sla1 clathin box (LLDLQ) does not contain an acidic residue in position 5. A GST fusion of Sla1 residues 798-813 that includes the LLDLQ variant clathrin box is capable of pulling down clathrin from S. cerevisiae extracts. Importantly, this interaction is negatively regulated by the Sla1 homology domain 2 (SHD2), a domain with no previously known function. Live cell imaging experiments indicate that both the LLDLQ variant clathrin box and the SHD2 domain are critical for endocytosis of GFP-tagged Wsc1, an NPFxD-dependent endocytic cargo. SHD2 was originally defined as a domain because of its sequence conservation within Sla1 proteins from different species. However, SHD2 does not have significant sequence homology to any known protein domain. This study presents the 1.9 Angstrom crystal structure of SHD2. The structure reveals a SAM (sterile alpha motif) topology, one of the most abundant protein-protein interaction motifs, with functional roles in myriad of biological processes from signal transduction to transcriptional and translational regulation. Our results uncover a novel clathrin binding regulation mechanism by SHD2. Its relationship with the SAM fold endows this superfamily with a new role in cell biology, the regulation of clathrin adaptor funtion.

928/B127

Cumulative Contributions of Ubiquitin Sorting Receptors and Trafficking of the Multivesicular Body Pathway.

S. Shields[1], A. Oestreich[2], S. Winistorfer[1], D. Katzmann[2], R. Piper[1]; [1]Molecular Physiology and Biophysics, University of Iowa, Iowa City, IA, [2]Biochemistry and Molecular Biology, Mayo Clinic, Rochester, MN

Ubiquitinated membrane proteins are transported into the interior of the lysosome for degradation. This sorting pathway involves incorporation of cargo proteins into endosomal intralumenal vesicles, which accumulate to form a multivesicular endosome/body (MVB). Some of the proteins that make up the sorting apparatus have been identified as the class E Vps proteins that associate in distinct ESCRT complexes that localize to the surface of endosomes. An important interaction for the proper sorting of ubiquitinated proteins is the ability of an early ESCRT complex to recruit clathrin to the surface of the endosome. Additionally, several of the individual ESCRT components can non-covalently bind ubiquitin and this has led to a model whereby ubiquitinated membrane protein cargo is sequentially recognized and moved by a series of ubiquitin-binding ESCRT proteins. We set out to test this model using available structural data that describes how individual ESCRT components bind ubiquitin. Interestingly, ablating the ubiquitin-binding capacity of known ESCRT-I and ESCRT-II components had little effect on MVB sorting, even though these mutants caused synthetic effects upon disruption of other interactions between ESCRT complexes or disruption of the ablility to bind clathrin. In a search for other candidate ubiquitin-sorting receptor components, we found a novel ESCRT-I protein that binds a novel surface of ubiquitin localized near the C-terminus. While ablation of its ubiquitin-binding ability had no effect on its own, it did have a synthetic effect on the efficiency of sorting ubiquitinated cargo when combined with mutations that alter the ubiquitin-binding capacity of other ESCRT components. In summary, we see a progressive loss in the efficiency of MVB sorting when mutations in individual components are combined indicating that the ESCRT complexes may act together at a single sorting step to recognize ubiquitinated cargo. Multiple ubiquitin binding sites may be in place to ensure that a wide variety of ubiquitinated proteins, which may present ubiquitin in different conformations, can be recognized by at least one ubiquitin-binding sorting receptor subunit.

929/B128

Genetic Studies on the Endocytic Pathway Member Snf7 Reveal New Complexities in Signal Transduction Regulation.

J. M. Wolf, D. A. Davis; Microbiology, University of Minnesota, Minneapolis, MN

Recent studies have shown that endocytosis contributes to many signal transduction pathways in the eukarya, including the Rim101/PacC signaling pathway in fungi, such as C. albicans. Snf7 plays a central role in endocytosis through its function in the endosomal sorting complex required for transport (ESCRT) pathway that generates multivesicular bodies prior to fusion with the vacuole/lysosome. Snf7 is also required for the activation of the transcription factor Rim101 by proteolytic processing. We investigated the role of Snf7 in these two processes, and hypothesized that Snf7 function in endosomal sorting is distinct from its function in Rim101 processing. To test this hypothesis, we generated a series of alanine scanning mutations and performed phenotypic analyses on strains expressing only the mutant snf7 allele. Mutant alleles could be categorized into four groups: 1, with no phenotypic defects; 2, with Rim101-specific defects; 3, with ESCRT-specific defects; 4, with defects in both processes. Snf7 is recruited to the endosomal membrane for both Rim101 and ESCRT processes, thus group 4 alleles could be defective in recruitment to the endosomal membrane or be defective in a downstream function common to both pathways. Fractionation studies revealed that group 4 Snf7 proteins were localized similar to wild-type Snf7, suggesting that group 4 alleles are defective for a downstream function of Snf7. Since Rim101 processing is required for Rim101 activation, group 2 alleles were predicted to be defective in Rim101 processing, similar to a snf7 null. Unexpectedly, we found that in many group 2 alleles Rim101 processing was similar to wild-type. Processed Rim101 localizes to the nucleus where it governs changes in gene expression; however, immunofluorescence analyses have revealed that in the group 2 alleles, Rim101 is localized in the cytoplasm. Thus, our results establish that mechanisms, in addition to processing, are required to localize processed Rim101 to the nucleus. One idea currently being addressed is a Snf7-dependent release of processed Rim101. These studies highlight and dissect the active involvement that the endocytic machinery plays in signal transduction in eukaryotic cells.

930/B129

Vps45p Binds to the Closed Conformation of Tlg2p.

M. L. Furgason[1], N. Bryant[2], M. Munson[1]; [1]Biochemistry & Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, [2]Biochemistry and Molecular Biology, University of Glasgow, Glasgow, United Kingdom

The Sec1p/Munc18 (SM) family of proteins regulates intracellular trafficking through interactions with their cognate SNARE proteins. Elucidation of a common mechanism of function has been difficult, largely due to the multiple modes of binding observed between different SM proteins and their partner(s). Here, we used quantitative in vitro binding assays to examine the interactions between yeast Vps45p (endocytic SM protein), Tlg2p (syntaxin family SNARE), and Tlg2p/Tlg1p/Vti1p/ Snc2p (SNARE complex). Previously, the binding of Vps45p to Tlg2p and SNARE complexes was proposed to be mediated solely through a conserved interaction with the N-terminal peptide of Tlg2p. Our studies have identified a hitherto uncharacterized Vps45p-Tlg2p interaction, analogous to the interaction observed between neuronal Munc18a and syntaxin 1A: Vps45p binds to the closed conformation of Tlg2p. These results unify the disparate models, by demonstrating that Vps45p uses all of the binding modes reported between SM proteins and their cognate syntaxins.

931/B130

Upgrade: A Novel Approach to Studying Ubiquitin Dependent Trafficking.

D. Stringer, R. Piper; University of Iowa, Iowa City, IA

Ubiquitination mediates sorting of integral membrane proteins to lysosomes for their degradation. While much is known about what ubiquitin can do for a subset of very well characterized model proteins, it is technically quite challenging to determine whether ubiquitin actually plays a role in regulating a particular protein of interest. Recently, we have developed a technique where we can target de-ubiquitinating enzymes to specific proteins, compartments, and sorting complexes. We find this technique affords exceptional specificity and can be used to functionally dissect the role of ubiquitination in the endocytic pathway. In particular, we have targeted de-ubiqutinating enzymes to the ESCRT (endosomal sorting complex required for transport) machinery, which moves proteins into the interior of the MVB (multi-vesicular endosome/body). Our evidence indicates that ESCRT subunits do not need to be ubiquitinated to sort membrane proteins into the MVB or to form intralumenal vesicles.

932/B131

SNX9 Regulates Tubular Invagination of the Plasma Membrane through the Interaction with Actin Cytoskeleton and Dynamin-2.

N. Shin, J. Park, C. Ha, Y. Kim, S. Lee, S. Chang; Life Science, Gwangju Institute of Science and Technology, Gwangju, South Korea

Dynamic membrane remodeling during intracellular trafficking is controlled by the intricate interplay between lipids and proteins. BAR domains are modules that participate in endocytic processes by binding and deforming the lipid bilayer. Sorting nexin 9 (SNX9), which functions in clathrin-mediated endocytosis, contains a putative BAR domain, however, the properties of this domain have been less investigated. Here we show that SNX9 shares many properties with other BAR domain-containing proteins, such as amphiphysin and endophilin. SNX9 is able to deform the plasma membrane, as well as liposomes, into narrow tubules and recruit N-WASP and dynamin-2 to these tubules via its SH3 domain. SNX9-induced tubulation is antagonized by N-WASP and dynamin-2 while it is enhanced by perturbation of actin dynamics. However, SNX9 also has several unique properties. The tubulating activity requires both its BAR and PX domains as well as the low complexity (LC) domain which binds the Arp2/3 complex. SNX9 also binds to PtdIns[4]P-5-kinases via its PX domain and its tubulating activity is regulated by phosphoinositides. In addition, the kinase activity of PtdIns[4]P-5-kinases is stimulated by interaction with SNX9, suggesting a positive feed back interaction between SNX9 and PtdIns[4]P-5-kinases. These results suggest that SNX9 functions in the coordination of membrane remodeling and fission via interactions with actin-regulating proteins, endocytic proteins and PtdIns[4,5]P2-metabolizing enzymes.

933/B132

GAP-43 Regulates the Free Calmodulin Level Necessary for Rapid Endocytosis in Chromaffin Cells.

E. Tisdale, F. Azizi, C. Artalejo; Dept. of Pharmacology, Wayne State University School of Medicine, Detroit, MI

Neuromodulin (GAP-43) is a major calmodulin (CaM) binding protein in neurons. It has been proposed that GAP-43 binds CaM and releases CaM locally at specific sites on the plasma membrane and thereby plays a key regulatory role in synaptic plasticity. However, the physiological significance of the interaction between GAP-43 and CaM in vivo and its relevance to neurotransmission is not known. In neurosecretory cells, vesicle exocytosis is followed by coupled endocytosis. Previously, we reported that adrenal chromaffin (AC) cells exhibit two kinetically and mechanistically distinct pathways of endocytosis that are coupled to different extents of exocytosis: rapid endocytosis (RE) is dynamin-1 dependent but clathrin-independent and slow endocytosis (SE) is dynamin-2 and clathrin-dependent. We have also shown that Ca2+ is unequivocally involved in RE, CaM is the Ca2+-receptor for RE, and replacement of extracellular Ca2+ by Sr2+ or Ba2+ completely blocked RE but had little effect on SE. In this study, we have investigated the role of GAP-43 on RE and SE under physiological conditions using capacitance and amperometry measurements. To ascertain whether GAP-43 was functionally involved in vesicle retrieval following exocytosis, we blocked function by introducing anti-GAP-43 antibodies into AC cells. These treatments abolished RE whereas there was no effect on SE, exocytosis or Ca2+ currents. Inclusion of exogenous CaM, dialyzed into the cell via the whole-cell patch pipette, rescued RE in cells treated with anti-GAP-43 antibodies. The results indicate that RE specifically requires CaM release from GAP-43 upon cell stimulation. Our data demonstrates that GAP-43 is part of the CaM- and dynamin 1-dependent molecular machinery that mediates rapid vesicle recycling. Therefore, GAP-43 is the prime candidate for regulating free CaM levels at endocytic sites. We propose that GAP-43 serves to sequester CaM at the inner face of the plasma membrane in close proximity to the CaM-dependent molecular machinery that mediates rapid recycling (RE) of vesicles

934/B133

Both BAR and F-BAR Domain Proteins Control Membrane Curvature and Vesicle Scission Generated by F-Actin Pulling Forces during Endocytosis.

T. Kishimoto[1], Y. Sun[1], R. Rounsevell[2], A. Michelot[1], C. Toret[1], D. Drubin[1]; [1]Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA, [2]Bioengineering, University of California, Berkeley, Berkeley, CA

Cell membranes change their shapes dynamically during endocytosis. Actin assembly plays a crucial function in membrane invagination during endocytosis in budding yeast. Several studies revealed that BAR and F-BAR (FCH and BAR) domain proteins participate in the regulation of membrane curvature during endocytosis. Many BAR and F-BAR domain proteins are also implicated in actin regulatory mechanisms, providing links between membrane invagination and the actin cytoskeleton. However, how BAR, F-BAR and actin functions cooperate to perform the temporal and spatial regulation of membrane invagination is not understood. Budding yeast BAR domain proteins Rvs167 and Rvs161 are involved in the membrane invagination and vesicle scission steps during endocyotosis. Although whether yeast F-BAR domain proteins are involved in membrane invagination is not known, the F-BAR protein Bzz1 does promote actin polymerization. We report here that Bzz1 cooperates with BAR domain proteins for efficient vesicle scission. The unique internalization defect seen for *rvs167* and *rvs161* mutants is exacerbated by *bzz1* mutations. Interestingly, the partial disruption of F-actin assembly caused a severe defect in vesicle scission in *rvs161/167* mutants independent of F-BAR dysfunction. These results suggest that F-actin pulling forces cooperate with BAR and F-BAR proteins to promote endocytic vesicle scission in budding yeast.

935/B134

Syp1p: a Conserved Component of the S. cerevisiae Endocytic Machinery.

H. E. Stimpson, C. Toret, B. S. Pauly, A. T. Cheng; Department of Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Receptor-mediated endocytosis is an essential eukaryotic process by which plasma membrane proteins are internalized. Although endocytic internalization has been extensively studied in real-time, we have little understanding of the mechanisms by which endocytic sites are initiated or by which cargo is selected. Proteins which arrive early at the endocytic site, are clear candidates for regulation of these processes. Here, we identify the protein Syp1p as a novel component of the early endocytic machinery in S. cerevisiae, and show that it interacts with the adapter protein Ede1p. Sequence alignments show that Syp1p has homology to the F-BAR domain at its N-terminus, and suggest that it may be part of a conserved family of proteins. Using real-time live-cell microscopy we show that GFP-tagged Syp1p is - like Ede1p- an early component of the endocytic site. Analysis of endocytic "patches" in yeast shows that Syp1p and Ede1p do not move into the cell with the internalizing vesicle. This suggests that Ede1p and Syp1p function in early endocytic steps such as cargo selection and patch initiation. In support of this theory, Z-stack analysis of various GFP-tagged patch components revealed that fewer endocytic patches are formed in the absence of Ede1p. We also show that Ede1p is required for recruitment of Syp1p to patches, and that Syp1p plays a role in determining the regions of the cell in which endocytic sites are formed. We are currently investigating the possibility that Syp1p and Ede1p may be involved in cargo selection as well as patch initiation.

936/B135

Novel Interactions of ESCRT-III with LIP5 and VPS4 and their Implications for Multivesicular Body Biogenesis.

S. Shim, S. A. Merrill, P. I. Hanson; Cell Biology and Physiology, Washington University, St. Louis, MO

The cellular machinery responsible for multivesicular body biogenesis is important for receptor downregulation, viral budding, and cell abscission. Endosomal sorting complex required for transport (ESCRT)-III is believed to drive these budding events, while the ATPase VPS4 reycles ESCRT-III with the aid of LIP5, a cofactor. Much is unknown about how these factors interact and how they participate in vesicle formation. Here we show that a subset of ESCRT-III proteins, including CHMP1B, CHMP2A/hVps2-1, CHMP3/hVps24, and CHMP5/hVps60 but not CHMP4/hSnf7-1 or CHMP6/hVps20 bind LIP5 with high affinity. Knock-down of LIP5 inhibits receptor downregulation, highlighting the importance of LIP5 function in this pathway. Analysis of the domains responsible for interaction of LIP5 with particular ESCRT-III proteins reveals that CHMP1B and CHMP2A bind LIP5 via C-terminal sequences that overlap with a MIT interaction motif (MIM) previously found to bind to VPS4. CHMP5 does not have the conserved MIM and binds LIP5 via a distinct motif. LIP5 contains two MIT domains near its N-terminus. Using site-directed mutagenesis, we show that the first (MIT1) is critical for interaction with CHMP2A and CHMP1B while the second (MIT2) is important for binding to CHMP5. These observations suggest that LIP5 may play a role in creating a high concentration of VPS4 on assembled ESCRT-III polymer. We also find evidence for a second binding site for VPS4 in CHMP2A and CHMP1B, suggesting that LIP5 and VPS4 may bind simultaneously to these proteins despite the overlap in their primary binding sites. Finally, LIP5 binds preferentially to soluble CHMP5 but instead to polymerized CHMP1B and CHMP2A, suggesting that the interaction between LIP5 and ESCRT-III proteins may be regulated by ESCRT-III conformation. This study adds new insight into how LIP5 and VPS4 cooperate in the pathway leading to multivesicular body formation.

937/B136

A Genome-Wide Killer Toxin Sensitivity Screen in Budding Yeast Reveals New Functions for Endocytic Patch Proteins.

S. Carroll, P. C. Stirling, D. Drubin; Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Similar to Shiga toxin and Cholera toxin, budding yeast K28 killer toxin depends on endocytosis to enter and ultimately kill cells. Indeed, as with some toxins that cause human disease, K28 is thought to be endocytosed, trafficked to the endoplasmic reticulum and then transported into the cytoplasm prior to exerting its toxic effects. For the present study we screened a library of *Saccharomyces cerevisiae* gene deletions to elucidate the full pathway of K28 binding, uptake, transport and killing. The screen identified deletions of 140 genes that result in partial or complete resistance to K28 toxin relative to wild-type strains, and deletions of 116 genes that result in toxin hypersensitivity. The resistant gene deletions cluster in protein complexes and pathways along the proposed route of toxin entry, while the hypersensitive deletions cluster in protein complexes and pathways that normally resist the uptake, trafficking and action of K28. These studies are the first to systematically identify determinants of K28 toxin sensitivity, ostensibly illuminating the entire pathway of toxin uptake, trafficking and killing. Furthermore, our data suggest functions for endocytic patch proteins for which functions had not previously been identified. Specifically, one class of endocytic patch protein appears to be essential for K28 internalization and/or trafficking, but not for fluid-phase endocytosis or for the internalization and/or trafficking of other cargos internalized via receptor-mediated endocytosis.

938/B137

Endosomal SNARE-dependent Inhibition of Phagocytosis by Aspirin in Dendritic Cells.

D. Cai, K. Chiow, Y. Ho, S. Wong; Department of Microbiology, National University of Singapore, Singapore

Acetylsalicylic acid (ASA) or Aspirin has been widely used as an analgesic, anti-inflammation, anti-pyretic and anti-thrombotic drug. Recent findings/reports showing its preventive effects on colon cancer and Alzheimer's disease arouse great interest to many researchers. The occurance of ASA resistance raised the possibility of utilizing high-doses of ASA in treating inflammatory related diseases. ASA and its metabolite, salicylate, were also reported to be able to modulate antigen presenting functions of dendritic cells (DC). However, the intracellular targets of ASA in DC are still poorly understood. Since phagocytosis is the initial step taken by antigen-presenting cells in the uptake of antigens for processing and presentation, ASA might exerts its immune-regulatory effects by regulating phagocytosis. Here, we showed that ASA, at low concentration (1-2 mM) enhances phagocytosis of E. coli by DC. However, at higher concentration (above 4 mM), ASA inhibits phagocytosis, instead. Furthermore, we have also observed that the protein expression of endosomal SNAREs was modulated upon ASA-treatment and the inhibitory effect of high-doses of ASA on bacteria up-take is dependent on the expression of at least two endosomal SNAREs. Thus, inferring that ASA, at high dosage exerts its phagocytic inhibitory effect by regulating the expression, and possibly intracellular localization of endosomal SNAREs in phagocytes.

939/B138

The Mechanism of Non-opsonized Phagocytosis of Silica Particles by Macrophages.

R. Gilberti, L. Costantini, D. Knecht; Molecular and Cell Biology, University of Connecticut, Storrs, CT

Inhalation of crystalline silica leads to the development of silicosis. We have demonstrated that cultured macrophages bind and internalize opsonized (antibody-coated) and non-opsonized (protein-coated or uncoated) silica and latex beads. The literature suggests that the negative surface charge of silica is important for receptor binding, yet the rate and extent of non-opsonized particle uptake is unaffected by the type of protein used for coating or particle charge. Interestingly, the rate of non-opsonized particle uptake is temperature-dependent. Uptake of non-opsonized particles is slower than that of opsonized particles at 25°C, but occurs at nearly the same rate at 37°C. This data suggests that macrophages internalize non-opsonized particles by a temporally, and possibly mechanistically, distinct pathway. The uptake of either crystalline or amorphous silica by macrophages induces cell death by 12 hours. In other cell types, silica particles are internalized, but there is no apparent toxicity. Surprisingly, directing opsonized silica to the macrophage Fc receptor pathway leads to a dramatic reduction in cell death. We hypothesize that suppression of cell death by opsonization of silica is due to differences in opsonized versus non-opsonized phagosomal trafficking. Molecular markers involved during Fc receptor-mediated uptake are being used to examine this novel route of entry. Macrophages expressing mCherry-actin extend actin-rich protrusions to engulf non-opsonized silica. When cells are pre-treated with actin polymerization or PI3 kinase inhibitors, there is minimal uptake of non-opsonized silica or latex beads. When Fc receptor-GFP expressing macrophages are exposed to opsonized silica or latex beads, Fc receptors become concentrated in the phagosomal membrane surrounding the particle. As expected, Fc receptors do not concentrate in non-opsonized particle phagosomal membranes. Comparable experiments are in progress using the putative silica receptors of the scavenger receptor-A family (SR-AI, II, MARCO). Investigation of non-opsonized phagosomal trafficking events using well-characterized markers of the Fc receptor pathway is currently underway. [Supported by Patterson Trust Foundation]

Protein Targeting to the Endocytic Pathway (940 – 951)

940/B139

Copper Toxicosis Gene COMMD1 Encodes a Novel, Oligomeric PtdIns(4,5)P2-Binding Protein Targeted to Endocytic Membranes.

J. L. Burkhead; Oregon Health & Science University, Portland, OR

Copper Metabolism Murr1 Domain 1 (COMMD1) is a 21 kDa protein involved in copper export from the liver, NF-κB signaling, HIV infection, and Na-transport. The precise function of COMMD and the mechanism through which COMMD1 performs its multiple roles are not understood. In order gain insight into the mechanism of COMMD1 function, we set out to identify the subcellular location of endogenous COMMD1 and investigate its biochemical properties. Recombinant COMMD1 is a soluble protein, yet in cells COMMD1 is largely seen as targeted to cellular membranes. Using co-localization with organelle markers and cell fractionation, we determined that COMMD1 is located in the vesicles of the endocytic pathway, while little COMMD1 is detected in either the trans-Golgi network or lysosomes. The mechanism of COMMD1 recruitment to cell membranes was investigated using lipid-spotted arrays and liposomes. COMMD1 specifically binds phosphatidylinositol PtdIns(4,5)P2 in the absence of other proteins and does not bind structural lipids; the phosphorylation of PtdIns at position 4 is essential for COMMD1 binding. Proteolytic sensitivity and molecular modeling experiments identified two distinct domains in the structure of COMMD1. Both domains appear to contribute to the lipid binding site, since only the full-length protein binds to PtdIns(4,5)P_2. In native conditions, endogenous COMMD1 forms large oligomeric complexes both in the cytosol and at the membrane; interaction with PtdIns(4,5)P_2 increases the stability of oligomers. Altogether, our results suggest that COMMD1 is a scaffold protein in a distinct sub-compartment of endocytic pathway and offer first clues to its role as a regulator of structurally unrelated membrane transporters.

941/B140

Retromer-dependent Endsomal Sorting of Mammalian Iron Transporter, DMT1.

M. Tabuchi, I. Yanatori, M. Ikebukuro, F. Kishi; Molecular Genetics, Kawasaki Medical School, Kurashiki, Japan

Divalent metal transporter 1 (DMT1) is indispensable molecule for both the dietary-iron absorption from apical plasma membrane in the duodenum and the iron acquisition from the transferrin-cycle endosomes in peripheral tissues. Previously we showed that two isoforms of DMT1, differing at their C termini (DMT1-I and -II) resulted from the alternative splicing of 3' exons, target to the distinct endosomal compartments and identified the Y^{555}XLX-sequence in the C-terminus of DMT1-II as the sorting signal for delivery to the transferrin receptor (TfR)-positive recycling endosomes. To examine the structural requirement for the appropriate sorting signal of DMT1-II, we

performed a detailed mutational analysis of this sequence. As a result, we have found that the sequence motif "TM-$_{(X)\geq 15}$ΦX(L/M)" (where X can be any residues, and Φ can be any hydrophobic residues) is structurally sufficient for the function of this sorting signal. Through the search for the known sorting motifs, we found the structurally similar sequence motif WLM, which has been recently identified in the cation-independent mannose-6-phoshate receptor tail as a retromer-mediated endosome-to-trans-Golgi network (TGN) retrieval. To investigate the potential role of retromer complex on DMT1-II-recycling endosome targeting, we have examined the effect of depletion of retromer function on DMT1-II localization. Depletion of one of the retromer subunits hVps35 by its siRNA results in missorting of the DMT1-II to late endosomes and lysososmes. On the other hand, TfR, which is normally colocalized with DMT1-II, appears to be invariably sorted to recycling endosomes. It indicates that DMT1-II uses the proper sorting pathway to get to the recycling endosomes, which is distinct from that of TfR. We also found that DMT1-II is partially colocalized with hVps35 in relatively large puncta corresponding to sorting endosomes. These data directly show that retromer is required for sorting of DMT1-II to recycling endosomes probably via the TGN. This sorting event must be of special importance for iron acquisition from transferrin-cycle endosomes during the iron recycling and the red blood cell maturation.

942/B141

Artificial Lipid-Anchored Proteins are Co-internalized with Endogenous GPI-proteins from the Plasma Membrane, Independently of 'Raft' Affinity.

J. R. Silvius[1], P. Bhagatji[1], J. Comeau[2], R. Leventis[1]; [1]Biochemistry, McGill University, Montréal, QC, Canada, [2]Chemistry, St. Francis Xavier University, Antigonish, NS, Canada

Glycosylphosphatidylinositol-anchored (GPI-) proteins are internalized mainly via a specialized clathrin- and dynamin-independent, cdc42- and Arf1-regulated pathway in various mammalian cell lines. To date, the physical and structural determinants of this distinctive endocytic routing of GPI-proteins have remained obscure, although mechanisms based on differential association of GPI-proteins with lipid-based membrane microdomains have been suggested. To explore this question we have used fluorescence microscopy and quantitative image analysis to compare the early endocytic routing in CHO cells of artificial lipid-anchored proteins, lipid markers and endogenous GPI- and transmembrane proteins. At early times after internalization, fluorescent proteins tethered to the cell membrane via phosphatidylethanolamine-polyethylene-glycol (PE-PEG) 'anchors' colocalize strongly with GPI-proteins but poorly with transferrin, a marker of clathrin-mediated endocytosis (CME), in peripheral endocytic vesicles. By contrast, bulk membrane lipid markers such as Bodipy-sphingomyelin, as well as PE-PEG 'anchors' not linked to proteins, are internalized predominantly by CME under the same conditions. Strikingly, cointernalization of artificial lipid-anchored proteins with GPI-proteins is independent of the degree of saturation of the 'anchor' acyl chains. These findings indicate that neither specific recognition of the GPI-anchor nor association with specific lipid microdomains is necessary for uptake of exofacial lipid-anchored proteins into the distinctive peripheral endocytic intermediates employed by GPI-proteins. Our results moreover suggest that in the absence of specific CME-targeting motifs, steric effects due to the protein moiety may promote exclusion of lipid-anchored exofacial proteins from the clathrin-mediated endocytic pathway and thereby favor their internalization by alternative pathways. (Supported by the Canadian Institutes of Health Research).

943/B142

Subunit Composition Requirements for Vps4-stimulated ESCRT-III Disassembly.

B. Davies[1], I. Azmi[1], M. Babst[2], D. Katzmann[1]; [1]Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN, [2]Biology, University of Utah, Salt Lake City, UT

Endocytosis and lysosomal targeting of plasma membrane components impact the composition of the cell surface, thereby influencing cellular physiology on numerous levels. Lysosomal degradation of transmembrane proteins is accomplished through their inclusion into intralumenal vesicles (ILVs) of a multivesicular body (MVB). MVB-lysosome fusion results in the delivery of cargo-containing ILVs to the hydrolytic lumen of the lysosome for degradation. Cargo entry into this pathway is regulated and depends upon the function of the endosomal sorting complexes required for transport (ESCRTs). ESCRT-I and -II exist as stable complexes transiently recruited to the site of MVB sorting, while ESCRT-III assembly coincides with endosomal recruitment. ESCRT-III assembly has been implicated in membrane deformation during ILV formation, and the activity of the AAA-ATPase Vps4 is required for ESCRT disassembly and completion of MVB sorting. Vps4 is a type 1 AAA-ATPase and functions as an oligomer with numerous ESCRT-III interaction surfaces (in the form of microtubule interacting and trafficking domains, MITs). In vitro reconstitution of ESCRT-III disassembly has been utilized to investigate the composition of the Vps4 oligomer. Reconstituted ESCRT-III disassembly depends upon ATP, Vps4, and Vps4 ATP hydrolysis, the MIT domain of Vps4, and ESCRT-III subunits containing MIT-interacting motifs (MIMs). Inclusion of ATPase-defective Vps4 subunits (Vps4E233Q) into the oligomer compromises Vps4 function. However, the dominant negative effect of Vps4 E233Q is dependent upon the MIT domain of Vps4. This suggests both that ESCRT-III engagement by a Vps4 subunit incapable of hydrolyzing ATP blocks function of the oligomer and that concerted ATP hydrolysis by all subunits is not required for Vps4 function. Loss of the MIT domain alone does not display a dominant negative phenotype, suggesting that function of the Vps4 oligomer does not require MIM-binding by all Vps4 subunits. These results suggest that Vps4 interaction with ESCRT-III via the MIT domain is linked to nucleotide hydrolysis by the MIM-engaged subunit to facilitate ESCRT-III release.

944/B143

Regulation of Protein Sorting in the Endosomal System.

C. Burd, T. Strochlic, J. Liu; Department of Cell and Developmental Biology, University of Pennsylvania School of Medicine, Philadelphia, PA

Plasma membrane (PM) localization of proteins reflects a steady state that arises from bidirectional transport between the PM and endosomes; after endocytosis, recycling pathways return cargo to the PM, while retention of cargo results in lysosomal degradation. We are investigating how the interplay of the endosomal sorting machineries determine cargo fate using the S. cerevisiae iron transporter, Fet3-Ftr1. Endocytic sorting of Fet3-Ftr1 is regulated by iron; in iron-starved cells Fet3-Ftr1 is internalized slowly and recycled, but when iron is

available the rate of Fet3-Ftr1 endocytosis increases and it is targeted to the lysosome-like vacuole and degraded. Using a Ftr1-GFP-based genetic screen, we discovered roles for the retromer complex (Vps5, Vps17, Vps26, Vps29, Vps35) and the sorting nexin, Snx3/Grd19, in recycling of Fet3-Ftr1; in these mutants Fet3-Ftr1 is delivered to the vacuole regardless of iron status. A recycling signal identified in Ftr1 is directly bound by Snx3 and chemical cross-linking shows that Snx3 associates with retromer, suggesting that Snx3 functions as a cargo-specific retromer adapter. Retromer-dependent sorting of another protein, Vps10, requires the sorting nexin Mvp1/Snx8, but not Snx3, suggesting that combinatorial interactions between retromer and sorting nexins underlie cargo-specific, retromer-dependent sorting. Iron-induced vacuolar turnover of Fet3-Ftr1 is defective in rsp5 ubiquitin ligase and ESCRT mutants which are deficient in ubiquitin-dependent multi-vesicular body (MVB) sorting; in these mutants Fet3-Ftr1 is recycled in the presence of iron. Although Ftr1 is ubiquitylated, iron does not enhance ubiquitylation and endocytosis of a Fet3-Ftr1 mutant lacking all 22 cytosolic lysine residues is stimulated by iron. Fluorescence microscopy experiments show that internalized Fet3-Ftr1 is delivered to endosomes labeled by Vps27 (the MVB sorting receptor), retromer, and Snx3, in the presence and absence of iron. Sorting of Fet3-Ftr1 into the MVB pathway requires ubiquitylation of Fet3 or Ftr1, and ESCRT proteins, suggesting that ubiquitylation of Fet3-Ftr1 at the endosome is tightly coupled to MVB sorting; we propose that the recycling machinery uncouples these processes.

945/B144

β-arrestin2 Directs Clathrin-mediated Endocytosis of TGFβ Receptors.

S. E. McLean, M. Bhattacharya, J. Di Guglielmo; Physiology and Pharmacology, University of Western Ontario, London, ON, Canada

The Transforming Growth Factor β (TGFβ) signalling pathway is necessary for a variety of normal cellular processes such as apoptosis, differentiation, matrix synthesis, epithelial to mesenchymal transition (EMT) and migration. This pathway is currently under intense study due to its roles in pathological conditions such as cancer and fibrosis. However, the distinct mechanisms involved in TGFβ receptor turnover and the effect on signal transduction have yet to be fully elucidated. The objective of this study is to identify the signal driving TGFβ receptors to undergo clathrin-mediated endocytosis, which promotes receptor signalling, vs. membrane-raft mediated endocytosis, which promotes receptor degradation. It is hypothesized that cell-surface proteins interacting with the TGFβ receptors will influence the partitioning of receptors into clathrin-mediated endocytic pathways. Using sucrose-density ultracentrifugation analysis, immunofluorescence microscopy and receptor degradation studies, we show that the association of type III TGFβ receptors with type II TGFβ receptors is able to increase the partitioning into non-membrane raft fractions. Furthermore, we have found that the type II TGFβ receptor is able to form a direct interaction with β-arrestin2, which may influence its endocytosis and have effects on downstream signalling events. As TGFβ receptor endocytosis and degradation have immediate effects on TGFβ signalling, the identification of the type III TGFβ receptor and β-arrestin2 as promoting clathrin-mediated endocytosis may help explain how this pathway may become dysregulated in pathological conditions.

946/B145

A Role for the Actin Polymerization Machinery in AP-1-Dependent Trafficking.

M. Anitei, C. Stange, I. Parshyna, B. Hoflack; Biotechnology Center, Dresden, Germany

By combining proteomic screens and in vitro liposome-based systems that recapitulate the fidelity of protein sorting in vivo, we have identified new molecules associated with AP-1/clathrin coats that regulate protein targeting to lysosomes. Low affinity membrane binding sites, minimally composed of ARF-1 GTPase, sorting signals in selected transmembrane proteins and phosphatidylinositol-4 phosphate, account not only for a selective AP-1/ clathrin coat assembly, but also for the recruitment of actin nucleation complexes and of molecules important for subsequent membrane fusion (e.g., Rab11, Rab14). Our current results indicate that the actin nucleation complex, exemplified by its components CyFIP1&2 that co-localize and associate with AP-1 in vivo, regulates the formation of trans-Golgi network-derived transport intermediates together with WASP/N-WASP and Rac1. Interference with CyFIP expression leads to a change in the steady-state distribution of the CI-M6PR, impairs transferrin recycling (but not its uptake), as well as retrograde transport from endosomes to the trans-Golgi network. Since AP-1s, CYFIP, and PAK3 (a kinase that regulates the CYFIP-dependent actin polymerization) are associated with the fragile chromosome X-linked mental retardation syndrome in humans, we hypothesize that a reduced efficiency in AP-1-dependent lysosomal targeting relying on actin-based movements may represent a molecular basis of this disease.

947/B146

ESCRT-I Regulates but is not Essential for Lysosomal Trafficking of Signaling Receptors.

A. Henry, J. Hislop, M. von Zastrow; Cell Biology, UCSF, San Francisco, CA

A fundamental question in cell biology is how signaling receptors are sorted to various destinations within the cell, with some destined to recycle back to the plasma membrane while others are targeted to the lysosome for degradation. We have addressed this issue by focusing on the largest family of signaling receptors, G-protein coupled receptors (GPCRs). While the molecular machinery that regulates sorting events remains largely unknown, ubiquitination is believed to play a role in sorting cargo to the lysosome. Previously, we have shown that the delta-opioid receptor (DOR) traffics to lysosomes and undergoes degradation when ubiquitination has been prevented, questioning the importance of ubiquitination in sorting cargo to the lysosome. Using a variety of methods including biochemical analysis, live-cell imaging, and manipulation by RNA interference, we confirm that DOR is ubiquitinated but does not require receptor ubiquitination to traffic to lysosomes. Interestingly, while both wildtype and an ubiquitination-defective receptor mutant require HRS and VPS4 for lysosomal proteolysis, the wildtype receptor is specifically dependent on the ESCRT-I component Tsg101 at a later stage in its proteolytic degradation. Taken together, these data suggest that ubiquitination and ESCRT-I play a regulatory but non-essential role in lysosomal trafficking of DOR.

948/B147

Deciphering EpoR Internalization: Roles of JAK2 and p85 Beta.

R. Sulahian, L. Huang; Cell Biology, UT Southwestern Medical Center at Dallas, Dallas, TX

Erythropoietin (Epo) and its receptor (EpoR) play an essential role in the survival, proliferation and differentiation of precursor red blood cells into mature erythrocytes. The EpoR lacks intrinsic tyrosine kinase activity essential to mediate downstream signaling cascades, instead,

it associates with Janus tyrosine kinase 2 (JAK2) which upon Epo binding is auto-phosphorylated and activated. This results in tyrosine phosphorylation of the receptor by JAK2 followed by the recruitment of SH2-domain containing proteins that will carry on the signaling event. Once activated, the receptor is internalized and degraded. In cells from primary familial and congenital polycythemia (PFCP) patients, the EpoR is mutated and displays increased sensitivity to Epo leading to erythrocytosis. Sustained downstream signaling following stimulation suggests a failure in receptor down-regulation. The mechanism of EpoR endocytosis and downregulation in normal or pathological conditions remains unclear. Here we demonstrate that JAK2 kinase activity is required for Epo-dependent EpoR internalization via clathrin mediated endocytosis. In addition, EpoR with all 8 tyrosines mutated to phenylalanines (F8) fails to internalize, and that Y429, Y431 and Y479 are required for this function. A candidate-based siRNA screen identified that p85 alpha and p85 beta, two regulatory subunits of the PI3 kinase, are important for Epo-induced EpoR internalization. We also show that p85 beta binds to wild-type EpoR but not F8 upon Epo stimulation. Consistently, a truncated mutant EpoR found in PFCP patients, which lacks all 3 important tyrosines for receptor internalization, does not bind p85 beta and fails to internalize. More importantly, the addition of Y429 and Y431 and surrounding residues to this mutant PFCP receptor restores p85 binding and also ligand-induced internalization, suggesting that p85 beta binds to Y429 and/or Y431. Because over-expression of the N- terminal SH2 domains from both p85 alpha and p85 beta act as dominant negative proteins and resulted in defects in EpoR internalization, we propose that p85 interacts with the EpoR upon Epo induction and recruit proteins in the endocytic machinery for receptor internalization.

949/B148

LDLR Recycling is Regulated by PLD-Generated Phosphatidic Acid Involving PKA Activity.

J. Jung[1, 2, 3], A. González[1, 2, 3]; [1]Inmunologia Clínica y Reumatología, Fac. de Medicina. P. Universidad Católica de Chile, Santiago, Chile, [2]Centro de Regulación Celular y Patologia and Centro de Envejecimiento y Regeneración, Fac. Ciencias Biológicas. P. Universidad Católica de Chile, Santiago, Chile, [3]MIFAB, Santiago, Chile

Several protein trafficking events depend on protein kinase A (PKA) activity. In particular, down regulation of basal PKA activity has been shown to induce endocytosis and intracellular accumulation of the epidermal growth factor receptor in recycling endosomes (Salazar et al. Mol Biol Cell, 2002). Phospholipase D (PLD) generated phosphatidic acid (PA), which has been widely involved in protein trafficking, is able to down regulate the cAMP/PKA system by increasing the activity of certain phosphodiesterases. Here we show that inhibitors of PKA activity, including H89 and myr-PKI, impair low density lipoprotein receptor (LDLR) recycling in HeLa cells, as revealed by biotinylation assays and indirect immunofluorescence. The LDLR became accumulated in recycling endosomes colocalizing with transferrin-Alexa. Inhibition of the enzyme phosphatidic acid phosphohydrolase by propranolol increases the PA levels and reproduces the effect of PKA inhibitors upon LDLR recycling. Primary alcohols that inhibit PA production by PLD abrogate the effect of propranolol. Taken together with the effect of PKA inhibitors shown here, and with previous evidence that PA decreases cAMP levels and PKA activity, these results indicate that PLD/PA signaling system can interfere with the LDLR recycling machinery most likely through a PA-mediated lowering of PKA activity (Financed by FONDAP grant# 13980001, Basal Financial Grant PFB12/2007 and Millenium Project, Ministerio de Planificación).

950/B149

Endosomal KRas Signaling en Route to Lysosomes.

A. Lu[1], M. Matsuda[2], O. Bachs[1]; [1]Cell Biology, University of Barcelona, Barcelona, Spain, [2]Pathology and Biology of Diseases, Kyoto University, Kyoto, Japan

Ras proteins are small GTPases involved in the regulation of important cellular functions such as proliferation, differentiation and apoptosis. Understanding intracellular trafficking of Ras proteins is crucial to identify novel Ras signaling platforms. In the present study, we report that EGF triggers KRas translocation onto endosomal membranes -independently of calmodulin and PKC phosphorylation- through a clathrin-dependent pathway. From early endosomes KRas, but not HRas or NRas, is sorted and transported to late endosomes and lysosomes. Using the Raichu-KRas probe we identified for the first time in vivo, active KRas on Rab7 late endosomes, eliciting a signal output through Raf1. On these late endosomes, we also identified the p14/MP1 scaffolding complex and activated Erk1/2. Abrogation of lysosomal function leads to a sustained late endosomal MAPK signal output. Altogether, this study reveals novel aspects on the KRas intracellular trafficking and signaling shedding new light on the mechanisms controlling Ras regulation in the cell.

951/B150

FIP1/RCP Binding to Golgin-97 Regulates the Transport of Membrane Proteins from Early Endosomes to Trans-Golgi Network.

j. jing[1], J. Junutula[2], C. Wu[3], a. peden[4], R. Prekeris[1]; [1]Cell and Developmental Biology, University of Colorado Denver, Aurora, CO, [2]Genentech Inc., South San Francisco, CA, [3]Pharmacology, University of Colorado Denver, Aurora, CO, [4]University of Cambridge, Cambridge, United Kingdom

Various Rab GTPases, including Rab11, are the master regulators of endocytic sorting and transport pathways. We and others have shown that Rab11 and its effectors (known as FIPs) are required for the endocytic proteins transport. We have previously shown that FIP1 (also known as RCP) is required for the sorting of membrane proteins to the recycling pathway in early endosomes. Here we used fluorescent and electron microscopy as well as various functional transport assays to show that FIP1/RCP is also required for the protein sorting to the retrograde transport pathways from early endosomes to the TGN. The knock-down of FIP1/RCP results in accumulation of TGN38 and Shiga toxin in early endosomes, while having no affect on the retrieval of manosidase-6-phosphate receptor from late endsosmes to TGN. To identify the molecular mechanism FIP1/RCP function we isolated FIP1/RCP binding proteins by immunoprecipitation with anti-FIP1/RCP antibodies followed by proteomic analysis. Using this approach we have identified Golgin-97 as a putative FIP1/RCP-binding protein. Since Golgin-97 is known to play a role in regulating retrograde proteins transport pathway, we tested whether interactions of FIP1/RCP with Golgin-97 regulates FIP1/RCP-dependent transport from early endosomes to TGN. Using biochemical and yeast two-hybrid assays, we characterized FIP1/RCP and Golgin-97 interaction, and have shown that FIP1/RCP, but not other FIPs, directly bind to Golgin-97. Deletion mutagenesis analyses indicate that the FIP1/RCP-binding domain is situated at the N-terminal side of the Golgin-97-GRIP domain (domain mediating Golgin-97 recruitment to TGN). Consistent with these results, over-expression or depletion of FIP1/RCP does not affect Golgin-97

localization, but instead regulates the targeting of retrograde transport vesicles to the TGN. In summary, we suggest that FIP1/RCP is involved in retrieval of membrane proteins from early endosomes to recycling and retrograde transport pathways. We also propose that FIP1/RCP binding to Golgin-97 is required for the tethering and fusion of early endosome-derived retrograde transport vesicles to the TGN.

Golgi to Cell Surface Transport (952 – 966)

952/B159

A Novel Trans-Golgi Network Sorting Mechanism for Basolateral Proteins.

L. N. Nejsum[1], J. Nelson[1,2]; [1]Biological Sciences, Stanford University, Stanford, CA, [2]Molecular and Cellular Physiology, Stanford University, Stanford, CA

The pathway of exocytosis in polarized epithelial cells, from the Trans-Golgi Network (TGN) to the apical and basolateral (BL) plasma membrane domains, is well described. However, it is not known whether different proteins destined for the same membrane domain are sorted into different or the same vesicles at the TGN. These sorting events are likely to be important for specifying the contents of vesicles delivered to the plasma membrane, and as sites for cellular regulation of homeostasis in response to physiological signals. We generated an MDCK cell line stably expressing two highly homologous BL proteins, aquaporin-3 (AQP3)-GFP and AQP4-Orange. 10 minutes after release from a TGN block, cells were fixed and analyzed by fluorescence microscopy. AQP3-GFP and AQP4-Orange were mostly observed in separate post-Golgi carriers (PGCs) and the Pearson's coefficient for co-localization was 0.5 indicating little co-localization. BL proteins with different structure and function, were also separated into different PGCs, like AQP3 and Vesicular Stomatitis Virus Glycoprotein (VSV-G, Pearson's=0.6) and AQP4 and VSV-G (Pearson's 0.6). However, when we compared VSV-G and Low Density Lipoprotein Receptor (LDL-R) we found a high degree of co-localization in PGCs (Pearson's 0.8). Since VSV-G and LDL-R have homologous BL sorting motifs, we tested if the sorting of BL proteins into PGCs is dependent on the sorting signal. We constructed a chimeric protein consisting of the cytosolic domains of AQP4, which contain the sorting signals for AQP4, fused to AQP3 (AQP434). The chimeric protein has the transport properties of AQP3, but the sorting signals of AQP4. When we compared the localization of AQP4 with that of AQP434, we found a high degree of co-localization of AQP4 and AQP434 in PGCs. When compared to AQP3 and AQP4, the Pearson's coefficient had shifted from 0.5 to 0.8, indicating that the BL sorting signal is essential for sorting of BL proteins into their specific PGCs. We are currently investigating the contents of the different PGCs to identify the molecular mechanisms that specify sorting motif specific protein sorting into PGCs, and examining whether different PGCs have specialized properties.

953/B160

Rab6 Interacts with Myosin Vb at the Level of Recycling Endosomes.

S. Lodeho, D. Tenza, G. Raposo, S. Miserey-Lenkei, B. Goud; Cell biology, Institut Curie, Paris, France

Rab6 is a small GTPase associated with the Golgi apparatus and the trans-Golgi network membranes. Two isoforms of Rab6, Rab6A and Rab6A', regulate a retrograde transport route from endosomes to the endoplasmic reticulum via the Golgi apparatus. Rab6 has also been implicated in the regulation of exocytic processes. A two-hybrid screen leads to the identification of Myosin Vb, a known partner of Rab11 implicated in the recycling to the plasma membrane, as a Rab6 binding protein. We show here that Rab6A and Rab6A' interact in vitro and in vivo with Myosin Vb, within a region different of the Rab11 binding domain. The overexpression of a Myosin Vb mutant leads to a recruitment of endogenous activated Rab6 on recycling endosomes. Moreover we show that some exocytic and endocytic cargos accumulate in recycling endosomes when Myosin Vb function is perturbed by the use of dominant-negative mutants. Our results point out for an interplay between Rab6 and Rab11 functions at the level of recycling endosomes.

954/B161

Myosin-Dependent Fission of Rab6-Positive Transport Intermediates from the Golgi Complex.

S. Miserey-Lenkei[1], S. Bardin[2], L. Daviet[4], B. Goud[3], A. Echard[5]; [1]Cell Biology, Institut Curie CNRS UMR144, Paris, France, [2]Cell Biology, Institut Curie CNRS UMR144, Paris, France, [3]Cell Biology, Institut Curie CNRS UMR144, Paris, France, [4]Hybrigenics, Paris, France, [5]Cell Biology, Institut Curie CNRS UMR144, Paris, France

The Rab6 GTPase regulates bidirectional trafficking at the Golgi interface in mammalian cells. Indeed, Rab6 controls both a retrograde transport pathway between endosomes and the endoplasmic reticulum via the Golgi complex and an anterograde pathway between the Trans Golgi Network and the plasma membrane. We previously showed that Rab6-positive transport intermediates (Rab6-ITs) move along the microtubule network in a bidirectional manner, but whether the Rab6 function depends on the actin cytoskeleton is unknown. In order to identify the molecular motors implicated in either the movement and/or the fission of Rab6-ITs, we systematically depleted dynein, kinesins or myosins by RNAi in Drosophila S2 cells and followed the Rab6-GFP dynamics using high-resolution time-lapse microscopy. We identified a myosin whose inhibition affected Rab6-ITs behaviour. Depletion of the corresponding myosin orthologue in human cells led to the formation of long unfissioned Rab6-positive tubes connected to the Golgi apparatus and a concomitant reduction in the number of Rab6-ITs. In those tubes, markers of the Rab6-regulated retrograde and anterograde pathways were present. Interestingly, Rab6 directly interacts with this myosin and controls its recruitment to the Golgi apparatus. We conclude that actin-based motors are implicated in the fission of Rab6-ITs from the Golgi complex.

955/B162

ARFGAP Regulates Retrograde Transport of Shiga Toxin.

Y. Shiba, L. Johannes; UMR144, institute curie/CNRS, Paris, France

The retrograde route links early endosomes to the trans-Golgi network (TGN), bypassing the late endocytic pathway. The bacterial Shiga and cholera toxins, that bind to the glycosphingolipids globotriaosyl ceramide (Gb3) and GM1 respectively, and the endogenous cargos TGN46 and mannose 6-phosphate receptor are known to use this pathway, but the molecular mechanisms underlying retrograde transport are still

poorly explored. ADP-ribosylation factors (ARFs) are ~20kDa GTP-binding proteins that are required for protein traffic at the level of the Golgi apparatus and early endosomes. In this study, we expressed mutants of ARF and protein fragments which bind to ARF-GTP to study ARF function in the retrograde route. Our results clearly indicate that ARF1, but not ARF6 is required for endosome-to-TGN transport of Shiga toxin. We downmodulated the expression of 6 different ARFGAPs whose activities are reported to be specific for ARF1, but not ARF6, and identified one of them as being critical for retrograde transport of Shiga toxin, using morphological and quantitative biochemical tools. In cells depleted for this protein, Shiga toxin remains in endosomes. In these cells, Cholera toxin also does not reach the Golgi, and the steady state localization of TGN46 and mannose 6-phosphate receptor are affected. These results clearly suggested that ARF1 and one of its ARFGAP regulators participate in retrograde sorting.

956/B163

A Role for Syt1p in Regulation of Arl1p-Mediated Vesicular Trafficking.

P. Tsai, K. Chen, F. S. Lee; Institute of Molecular Medicine, Taipei, Taiwan

Arl1p is a member of the Arf-like protein (Arl) subfamily of small GTPases and is implicated in regulation of Golgi structure and function. Arl1p can cycle between GDP-bound and GTP-bound forms, however the molecular mechanisms involved in regulating the exchange of GDP for GTP have not been established. In this study, we demonstrate that yeast Syt1p, a guanine nucleotide-exchange factor (GEF) for Arf proteins, exhibits GEF activity toward Arl1p, but not Arl3p, Arf1p, or Arf2p. Arl1p interacts with Sec7 domain of Syt1p in a GTP-dependent manner. Syt1p, like Arl1p, localizes at trans-Golgi and specifically participates in the transport of a glycosylphosphatidylinositol (GPI)-anchored plasma-membrane-resident protein Gas1p from the trans-Golgi to the plasma membrane. In syt1 mutant cells, the fraction of Arl1p in trans-Golgi relative to cytosol was much less than it was in wild-type cells. Increasing Syt1p levels returned the distribution toward that of wild-type cells. Like Arl1p, Syt1p influences the distribution of Imh1p, an Arl1p effector. We conclude that Arl1p is in a dynamic equilibrium between trans-Golgi and cytosol, and the localization of Arl1p with trans-Golgi requires the exchange of GDP for GTP, which is accelerated by Syt1p.

957/B164

Snapin Plays a Key Role as an Adaptor for CPE Cytoplasmic Tail to Connect Hormone Vesicles to Microtubule Motor Complex.

J. Park, Y. Loh; Section on Cellular Neurobiology, National Institute of Health, Bethesda, MD

Regulated secretion of neuropeptides from neuroendocrine cells is important to maintain caloric homeostasis and synaptic transmission. Peptidergic vesicles containing the neuropeptides should be selectively targeted to the sites for the activity-based secretion. In our previous studies, the cytoplasmic tail of carboxypeptidase E(CPE) plays a crucial role as a vesicular receptor to recruit a dynactin/kinesins onto the vesicles containing proopiomelanocortin(POMC) in anterior pituitary cells and the others containing pro-brain-derived neurotrophin factor(proBDNF) in hippocampal neurons. POMC is a precursor protein of adrenocorticotrophin(ACTH), the anti-depressant, and melanocyte-stimulating hormone(MSH), the appetite controller, while BDNF from proBDNF is critical for intensification of synaptic transmission. Our yeast two-hybrid study using CPE tail as bait yielded snapin which directly interacted with the CPE tail. GST-tagged snapin pulled down the same set of microtubule motor proteins as the CPE tail did and excessive GST-snapin interfered with the interaction between CPE tail and the motor proteins. Overexpression of snapin decreased not only normal accumulation of POMC/ACTH vesicles in the processes but also the velocity and processivity of POMC/ACTH vesicle movements in live cells to the similar extent to overexpression of CPE tail, suggesting a functional interaction between CPE tail and snapin. siRNA-based knockout of snapin completely blocked regulated secretion of ACTH from K+/Ba2+-treated AtT20 cells while it did not affect the metabolism of cytoplasmic and intragranular proteins. Furthermore, we identified two different snapin domains responsible for binding to the CPE tail and microtubule motor complex by in vitro GST pulldown assay. The CPE tail-interacting domain could target its conjugated quantum dots to the outer surface of POMC/ACTH vesicles in vivo. Thus, the CPE tail/snapin-based system may selectively recruit dynactin-based motor complex to target the CPE-binding neuropeptides, such as POMC, pro-insulin, proenkephalin, and proBDNF, to the specific secretory sites to maintain proper regulated secretion for caloric homeostasis and brain function.

959/B166

Functional Analysis of Golgi-localized Rhomboid Proteases in Fission Yeast.

N. Tanaka[1], D. Shibutani[1], K. Nakai[1], K. Takegawa[1,2]; [1]Kagawa University, Kagawa, Japan, [2]Kyushu University, Fukuoka, Japan

Rhomboid proteins, which only discovered to be novel proteases in 2001, contain multiple membrane-spanning segments, and their catalytic residues are found within these hydrophobic domains. They cleave transmembrane domains of substrates within the lipid layer. Rhomboid proteins are conserved in all kingdoms of life, and function in diverse processes including quorum sensing in bacteria, mitochondrial membrane fusion, apoptosis, and stem cell differentiation in eukaryotes. Golgi-localized rhomboid proteins are also conserved in eukaryotes, but its role remains unclear, except for the primary regulator of EGF receptor signaling in *Drosophila*. We found homologues of rhomboid family proteases (*rob1*$^{+}$, *rob2*$^{+}$) from fission yeast *S. pombe* genome database. The hydropathy profiles of Rob1 and Rob2 predict a six-transmembrane helical structure with deduced protease active site (GXXG) in helix 4. When expressed under their own promoter in *S. pombe*, both Rob1-GFP and Rob2-GFP localized to the Golgi apparatus. *rob1*Δ cells showed zinc sensitivity and extensive missorting of carboxypeptidase Y, which is known to be localized in vacuoles, to the cells surface. *rob2*Δ cells showed sensitivity to monensin, an ionophore that interferes with intracellular transport. These results suggest that Rob1 and Rob2 proteins play a distinct role at the Golgi and are involved in zinc homeostasis and protein sorting in *S. pombe*.

960/B167

The Exomer Coat Complex Transports Fus1p from the Trans-Golgi Network to the Cell Surface via a Novel Plasma Membrane Sorting Signal in Yeast.

R. Barfield, R. Schekman; Molecular and Cell Biology, University of California Berkeley, Berkeley, CA

Sorting of transmembrane cargo proteins to different cellular compartments is mediated by cytosolic sorting signals that are recognized and bound by coat proteins to recruit the cargo into vesicles. We have identified a region of the yeast cell fusion protein, Fus1p, that contains a sorting signal required for its transport to the plasma membrane. Fus1p is a single pass transmembrane protein that localizes to the plasma membrane in a polarized manner during the yeast mating response and is required at the cell surface during mating for cell fusion to occur. Transport of Fus1p to the cell surface is dependent on Chs5p, a component of the multisubunit exomer coat complex important for transporting cargo from the TGN to the plasma membrane in yeast. We show that Fus1p transport is also dependent on the exomer components Bch1p and Bud7p. Fus1p physically interacts with exomer and is retained at the TGN in the absence of CHS5 or BCH1 and BUD7. Mutations in the clathrin adaptor complex AP-1 restore Fus1p localization to the cell surface in the absence of exomer, suggesting that AP-1 is important for the intracellular localization of Fus1p. Deletion of a small region in the cytosolic domain of Fus1p abolishes its physical interaction with exomer, results in mislocalization of Fus1p, and therefore causes a cell fusion defect. Mutations in AP-1 allow this deletion construct to access the cell surface and rescue the cell fusion defect caused by this deletion. We suggest that this region of Fus1p contains a sorting signal that is recognized and bound by exomer leading to its capture into exomer coated vesicles and its transport to the cell surface. Because this region shows little or no similarity to other traffic signals, it appears to represent a novel sorting signal important for the transport of a cargo molecule to the plasma membrane in yeast.

961/B168

Visual Screening for Kinase-Regulated Membrane Trafficking Between Endosomes and Golgi: Identification of the Kianses That Involved In Extensive β-Amyloid Secretion.

A. Adachi[1], F. Kano[1,2], M. Murata[1]; [1]Depertment of Life Sciences, The University of Tokyo, Meguro-ku, Japan, [2]PRESTO, Japan Science and Technology Agency, Kawaguchi, Japan

Membrane trafficking is an important cellular process for the precise localization of membrane proteins. Disturbances in localization, in almost all cases, can disrupt protein function. Therefore, the disturbance of membrane trafficking induces various disease states. However, it is difficult to systematically explore the causal protein that induces the disturbances in spatially-regulated membrane trafficking. By developing an automated high-throughput fluorescence-based imaging system, we performed visual screening for kinases that regulate the localization of the cation-independent mannose 6-phosphate receptor (CI-M6PR) in HeLa cells. CI-M6PR is a membrane protein that cycles between the trans-Golgi network (TGN) and endosomes to deliver hydrolases to the lysosome, and its trafficking defects can cause many pathogenic states in cells; for example, Niemann-Pick disease, I-cell disease, and in particular, Alzheimer's disease (AD). As the result of our comprehensive visual screening to examine the effect of kinase inhibitors and a kinase siRNA library, we identified five kinases that regulate CI-M6PR trafficking. Moreover, we focused on AD to study the relationship between the five kinases and a disease state. Notably, the knockdown of two of the five kinases induced high levels of secretion of Aβ1-40 and Aβ1-42, as the hallmark of AD. These results showed that the repression of the two kinases is a risk factor of AD, and our study provides new approaches for the analysis of spatially-regulated membrane trafficking and related disease states.

962/B169

PH Domain-Dependent, Selective Transport of p75-NTR by Kinesin-3 Family Members in Non-Polarized MDCK Cells.

X. Xue, G. Kreitzer; Cell and Developmental Biology, Weill Medical College of Cornell University, New York, NY

During differentiation, epithelial cells develop polarized transport routes from the Golgi that promote proper targeting of membrane proteins to distinct apical and basolateral membrane domains. To do this, the machinery involved in selective trafficking must be regulated during polarization. We showed previously that KIF5B transports the neurotrophin receptor p75 selectively in polarized MDCK cells, but not in non-polarized cells. To identify the kinesin responsible for p75 trafficking in non-polarized MDCK cells, we expressed KIF-specific dominant-negative constructs and assayed for alteration of p75 transport using live cell imaging. Over-expression of the tail domains of Kinesin-3 family members that contain a pleckstrin homology (PH) domain (KIF1A or KIF1B β) inhibits export of p75 from the Golgi in non-polarized , but not in polarized MDCK cells. Expression of either the KIF1A tail deleted of the PH domain or the tail domain of Kif1B α, a KIF1B splice variant lacking a PH domain, has no effect on p75 trafficking. RNAi-mediated knockdown of KIF1A also inhibited post-Golgi transport of p75 in non-polarized MDCK cells. KIF1A appears to be a selective transporter since expression of KIF1A dominant negatives had no effect on post-Golgi trafficking of other apical membrane proteins in non-polarized cells. Immunofluorescence analysis revealed that endogenous KIF1A co-localizes with vesicles containing p75-containing vesicles in non-polarized MDCK cells. Together, our data suggest that Kinesin-3 motors transport p75 selectively in non-polarized MDCK cells in a PH domain-dependent manner.

963/B170

Lysozyme Trafficking in Macrophages.

C. Offenhäuser[1], M. Desclozeaux[1], T. Khromykh[1], J. Venturato[1], S. Gordon[2], J. Stow[1]; [1]Institute of Molecular Bioscience, University of Queensland, Brisbane, QLD, Australia, [2]Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

Lysozyme is a small protein with antibacterial and antiinflammatory properties. Lysozyme is secreted abundantly and constitutively by macrophages but the pathway for its release is largely unknown. We localized endogenous lysozyme in the secretory pathway in macrophages in late endosomes (LE) where it colocalized with various markers. Studies with Brefeldin A and temperature block experiments show that lysozyme is trafficked from the Golgi to LEs. Surface delivery of LAMP-1 and secretion of lysozyme into the medium suggest secretory lysosomes as the pathway for the release of lysozyme. Lysozyme is also delivered in LEs to phagosomes in these cells. Our results serve to characterize the molecules and organelles involved in lysozyme trafficking and immune responses in macrophages.

964/B171

Silencing of Trafficking Machinery In Vivo Demonstrates an Essential Role of a Trans-Golgi Network Golgin in the Regulated Secretion of TNFα By Activated Macrophages.

Z. Lieu[1], J. G. Lock[2], L. Hammond[2], N. L. La Gruta[3], J. Stow[2], P. Gleeson[1]; [1]Biochemistry and Molecular Biology and Bio21 Molecular

Science and Biotechnology Institute, The University of Melbourne, Melbourne, VIC, Australia, [2]Institute for Molecular Bioscience, University of Queensland, Brisbane, QLD, Australia, [3]Microbiology and Immunology, University of Melbourne, Melbourne, VIC, Australia
Understanding the role of trafficking machinery in vivo requires the ability to dissect transport pathways in a range of specialised cell types. Here we have employed RNAi to silence genes in cultured cells and whole animals to analyse the export of tumour necrosis factor-α (TNF). The transmembrane precursor of TNFα exits the trans-Golgi network (TGN) in tubular carriers for subsequent trafficking and delivery to the cell surface. Members of the TGN golgin family are associated with subdomains and tubules of the TGN and regulate individual transport pathways(1). In particular, TNFα transport from the TGN is mediated selectively by tubules and carriers marked by the TGN golgin, p230/golgin-245 (p230). Moreover, p230 is essential for intracellular trafficking and cell surface delivery of TNFα in transfected HeLa cells and activated RAW264.7 macrophages(2). Depletion of p230 in LPS-stimulated macrophages reduced cell surface delivery of TNFα by >10 fold compared with control cells. To determine if depletion of p230 blocked TNFα secretion in vivo, we have silenced p230 in animals by the generation of retrogenic mice expressing a micro RNA-vector to p230. Bone marrow stem cells were transduced with recombinant retrovirus containing micro RNA constructs and transplanted into irradiated recipients. Expression of the micro RNA construct did not perturb normal lymphoid and myeloid development pathways. Peritoneal macrophages from p230 miRNA retrogenic mice were depleted of p230 and upon LPS-activation had dramatically reduced levels of cell surface TNFα compared with macrophages from normal mice. Overall our studies have identified the TGN golgin p230 as a key regulator of TNF secretion. Moreover, we have demonstrated a novel approach to control cytokine secretion by the specific silencing of trafficking machinery. (1) Lock, JG, Hammond, LA, Houghton, F, Gleeson, PA and Stow, JL (2005) Traffic 6:1142 (2) Lieu, ZZ, Lock, JG, Hammond, LA, La Gruta, NL, Stow JL, and Gleeson, PA (2008) PNAS 105:3351

965/B172

A Role for Phosphoinositides in Cytokine Secretion.

R. Low[1], R. Misaki[1], T. Taguchi[1], F. A. Meunier[2], J. Stow[1]; [1]Institute of Molecular Bioscience, Brisbane, QLD, Australia, [2]Molecular Dynamics of Synaptic Function Laboratory, The Queensland Brain Institute, St Lucia, QLD, Australia
Activated macrophages secrete cytokines including tumor necrosis factor alpha (TNF). We previously defined a novel biosynthetic route for TNF that transits the recycling endosome. Here, we screened for a potential role of a class of membrane lipid enzymes, phosphoinositide 3-kinases (PI3Ks) in TNF trafficking. Using broad-class and isoform-specific inhibitors, a role for PI3K specifically its delta (δ) isoform, is indicated in TNF trafficking and secretion in macrophages. In particular, imaging of fixed cells and live-cell imaging revealed that TNF containing tubules are more prevalent and sustained at the trans-Golgi network (TGN) in the presence of the inhibitors compared to controls. Depleting PI3Kδ was found to reduce TNF surface delivery and confirmed the results with inhibitors. Our preliminary results from an in vitro budding assay suggest that PI3kδ may in fact peripherally associate with the budding carriers. Taken together, these findings suggest a novel function for PI3Ks in constitutive secretion and in particular for the post-Golgi transport of inflammatory cytokine TNF in activated macrophages.

966/B173

Functional Analysis of Axonal Transport in the Squid Giant Axon: Synergies and Competitions between APP and JIP1/2 for Kinesins.

D. Nobrega[1,2], A. Chirila[1], E. Bearer[1,2]; [1]Dept of Pathology, Brown University, Providence, RI, [2]Marine Biological Laboratory, Woods Hole, MA
Intracellular cargo attach to cellular transport machinery for targeted delivery throughout the cell. The squid giant axon serves as a powerful tool for transport studies because of the high degree of conservation of the transport machinery and the large size of the axon (0.6mm), which allows engineered cargo to be injected and its transport imaged. Using this model we discovered a 15-amino acid peptide from amyloid precursor protein (APP) sufficient to carry peptide-conjugated 100-nm fluorescent beads towards the synapse at fast axonal transport rates (Satpute et al. PNAS 2006). JIP1/2, a peripheral membrane scaffolding protein, is predicted to facilitate interactions between APP and kinesin (Verhey et al. JCB, 2001). Here, we test whether JIP1/2 mediates transport of exogenous cargo and compare transport of JIP1/2-, APP- and negative-charge-beads. Beads conjugated to JIP1/2 (aa 698-711), the KLC1 binding domain, transported rapidly (0.46±0.167 µm/s, n=357) at rates comparable to APP (0.41±0.18 µm/s, n=433) or carboxylated beads (0.35±0.16 µm/s, n=413). Peptides with jumbled sequences for JIP1/2 and APP, or a JIP1/2 peptide with a single mutation in the kinesin binding domain, moved rarely. Both JIP1/2- and APP-conjugated beads competed with carboxylated beads for transport. When unconjugated and peptide-conjugated beads were co-injected, the peptide-coated beads sustained movement (APP-C, 89.5±1.27% early and 83.8±1.5% later; JIP 90.8±2.5% early and 83.3±1.4% later) whereas unconjugated did not (37.2±24.9% early versus 4.7±1.8% later). When JIP1/2- and APP-beads were co-injected, JIP-beads retained robust transport, with 80% of beads moving, whereas APP-bead movements dropped to 40%. Soluble JIP peptide inhibited JIP-beads completely and APP-beads by 80%, whereas APP peptides only inhibited APP-beads. Blots of peptide pulldowns revealed that kinesins1, 2 and 3 bind differently to each peptide. Thus the designer-bead strategy to identify transport machinery in this functional assay produces information regarding minimal peptide "zipcodes" for recruitment to cargo of anterograde motor machinery and reveals competitions between cargo-receptors for motors and transport. Supported by NINDS NS046810(ELB).

Cytoskeleton-Membrane Interactions (967 – 992)

967/B175

Evidence of Direct MinE-Membrane Interaction and Its Involvement in Membrane Remodeling During Cytokinesis in E. coli.

Y. Shih[1,2], C. Lin[1], H. Lai[2,1], C. Hsieh[1]; [1]Institute of Biological Chemistry, Academia Sinica, Taipei, Taiwan, [2]Institute of Biochemical Science, National Taiwan University, Taipei, Taiwan
The cell division site placement in E. coli is mediated by the Min system. The Min system oscillates between two ends of a cell to allow site-specific formation of the division septum. The dynamic movement of the Min system is underlay MinE-regulated assembly and disassembly

of the MinD protein filaments on the cell membrane. In the previous model, MinE is proposed to be recruited to the membrane sites by MinD and several questions remain including mechanism of the MinE ring formation at midcell. Our study shows that MinE is capable of direct interaction with liposomes reconstituted from E. coli phospholipids in vitro. The MinE-membrane interaction takes place through electrostatic attraction between the positively charged N-terminal domain of MinE and the anionic phospholipids in the membrane. The N-terminal domain of MinE is also known to interact with MinD directly, thus selective binding of this domain may play an important role in regulating oscillation of the Min system. The moderate MinE-membrane interaction can further lead to deformation of liposomes, resembling that caused by proteins involved in vesicle formation in eukaryotic cells. Furthermore, MinE appears to organize into filamentous structures on the membrane surface that may correlate to the MinE ring formation inside cells. Evidences of the direct MinE-membrane interaction and self-organization of MinE into filamentous structures on the membrane provide new insights into the molecular mechanism of the Min system. In addition, the membrane-deforming properties of MinE and MinD are hinting at involvement of the Min system in membrane remodeling processes during cytokinesis in E. coli.

968/B176

Spatial Regulators for Bacterial Cell Division Self-Organize into Surface Waves In Vitro.

M. Loose[1,3], E. Fischer-Friedrich[2], J. Ries[1], K. Kruse[4], P. Schwille[1,3]; [1]Biotec, TU Dresden, Dresden, Germany, [2]Max-Planck-Institute for Physics of Complex Systems, Dresden, Germany, [3]Max-Planck-Institute for Molecular Cell Biology and Genetics, Dresden, Germany, [4]Theoretische Physik, Universitaet des Saarlandes, Saarbruecken, Germany

In the bacterium Escherichia coli, the Min proteins oscillate between the cell poles to select the cell center as division site. This dynamic pattern has been proposed to arise by self-organization of these proteins, and several models have suggested a reaction-diffusion type mechanism. Here, we found that the Min proteins spontaneously formed planar surface waves on a flat membrane in vitro. The formation and maintenance of these patterns, which extended for hundreds of micrometers, required ATP, and they persisted for hours. We present a reaction-diffusion model of the MinD and MinE dynamics that accounts for our experimental observations and also captures the in vivo oscillations.

969/B177

Dynamic Regulation of the Multidrug Efflux Transporter Sp-ABCB1a in Sea Urchin Embryos: How Location Along Microvilli Regulates Activity.

A. Hamdoun[1], D. Epel[1], S. Setayeshgar[2], X. Song[2]; [1]Hopkins Marine Station, Stanford University, Pacific Grove, CA, [2]Deprtment of Physics, Indiana University, Bloomington, IN

In embryonic development the structure of cell surfaces in general, and cortical actin structures in particular, are highly dynamic and undergo major reorganization following fertilization and during subsequent development. This study examines the relationship between changes in the activity of the multidrug efflux transporter Sp-ABCB1a (an ortholog of mammalian Permeability-glycoprotein) and changes in its location on cortical surfaces. Thirty minutes after fertilization there is a large (20-fold) increase in the activity of Sp-ABCB1a that ensues from redistribution of stored protein. Using a polyclonal antibody we find that most of the transporter in the unfertilized egg localizes to 2 µm beneath the surface, with only 10% on the egg's surface. After fertilization, there is an elongation of microvilli and 75% of the cortical transporter stores redistribute to the top 1 µm of the 2 µm long microvilli. To investigate the functional significance of this post-fertilization redistribution, we generated a mathematical model of efflux transporter efficiency as a function of location along microvilli. The model predicts that tip localized transporters are more efficient than evenly distributed transporters in scenarios where diffusion in the extracellular space between microvilli is slowed to 1 µm/sec, possibly by presence of an extracellular matrix layer (hyaline). Later in development there is a potent down-regulation of Sp-ABCB1a activity in the small micromeres. We found that over-expression of Sp-ABCB1a:mCherry increases PSC833 and cylcosporin-sensitive efflux activity 5 fold, yet the transporter inactivation in the small micromeres is potent enough to down-regulate both endogenous and over-expressed protein. We are presently examining the relationship between surface structure and activity in these cells. The broader significance of this study is in uncovering how the organization and structure of the cell's surface regulates the activity of membrane transporters.

970/B178

Molecular Mechanisms of Membrane Deformation by I-BAR Domain Proteins.

J. Saarikangas, H. Zhao, A. Pykäläinen, P. Laurinmäki, P. Mattila, P. Kinnunen, S. Butcher, P. Lappalainen; University of Helsinki, Helsinki, Finland

Membrane remodeling by proteins that directly sculpt biological membranes is an emerging field in cell biology. Among the central regulators of membrane dynamics are the BAR superfamily domains, which deform plasma membrane to tubular structures through their intrinsic curvature. In contrast to the relatively well characterized BAR and F-BAR domains that promote the formation of plasma membrane invaginations, the I-BAR domains (inverse-BAR, also known as the IRSp53/MIM domain; IMD) induce plasma membrane protrusions through a poorly understood mechanism. By applying cryo-electron microscopy and time-lapse imaging of giant unilamellar vesicles, we show that I-BAR domains bind to the inner leaflet of the membrane tubules and thus induce the formation of dynamic membrane protrusions to the opposite direction than the BAR and F-BAR domains. Our results also demonstrate that I-BAR domains induce strong clustering of PI(4,5)P2 trough electrostatic interactions. Comparison of different I-BAR domains revealed that they deform PI(4,5)P2-rich membranes through partially distinct mechanisms and consequently induce the formation of membrane tubules with different diameters and dynamics. Whereas the I-BAR domains of mouse IRSp53 and IRTKS bind PI(4,5)P2-rich membranes mainly through electrostatic interactions, the I-BAR domains of mouse MIM and ABBA also insert an amphipathic helix into the membrane. Consequently, MIM and ABBA domains induce the formation of thicker membrane tubules than the IRSp53 and IRTKS domains, and also display stronger filopodia-forming activity in vivo. Finally, our FRAP analysis revealed that while the membrane-bound I-BAR domains of IRSp53 and MIM exchange rapidly with the soluble pool in cells, the I-BAR domain of a C.elegans protein displays stable association with filopodia. Together, these data define I-BAR

domain as a novel functional member of the BAR superfamily, elucidate the mechanism by which I-BAR domains induce the formation of membrane protrusions in cells, and reveal a divergence in the mechanism of membrane deformation within this sub-family.

971/B179

Tropomodulin1 is Required to Maintain Fiber Cell Shapes and Ordered Hexagonal Packing in the Mouse Lens.

V. M. Fowler, R. Nowak; Cell Biology, the Scripps Research Institute, La Jolla, CA

Tropomodulin1 (Tmod1) binds tropomyosin (TM) and caps the pointed ends of actin filaments in the spectrin-based membrane skeleton underlying the plasma membrane. Tmod and the membrane skeleton have been implicated in morphogenesis and stability of the lateral membrane of cultured epithelial cells, but their roles in epithelial cell shape and packing organization in organogenesis have not been studied. The stereotyped program of vertebrate lens fiber cell morphogenesis in time and space provides a unique system to study the generation and maintenance of epithelial cell shapes and hexagonal packing geometry in an organ in vivo. We analyzed the consequences of loss of Tmod1 in the mouse lens. Lens fiber cell morphology and packing organization, Tmod1, TM, F-actin and spectrin localization were analyzed by immunofluorescence staining and confocal microscopy of equatorial cryosections, revealing lens fiber cell cross-sectional profiles. In the absence of Tmod1, initial morphogenesis of fiber cell hexagonal shape and packing is normal, but this is not maintained and cell shapes become abnormal with disordered packing as fiber cells mature and move inwards. Western blotting shows levels of a short, membrane-associated γ-TM are decreased dramatically, but levels of a long TM are unchanged. While global and membrane-associated F-actin and β2-spectrin levels are unchanged, quantitative fluorescence image analysis shows that both F-actin and β2-spectrin organization are perturbed on fiber cell membranes, even before defects in hexagonal packing are apparent. We conclude that Tmod1 capping is required to selectively stabilize short γ-TM-coated actin filament linkers in the spectrin-based membrane skeleton, thereby maintaining its uniform long-range organization in the plane of the membrane. The mechanical stability and elasticity conferred by the membrane skeleton network on epithelial cell membranes may be essential to maintain normal cell morphology and hexagonal packing interactions in vivo.

972/B180

F-Actin Is Essential To Stabilize IRSp53-IMD-Induced Filopodia.

C. Yang, T. Svitkina; Biology, University of Pennsylvania, Philadelphia, PA

Leading edge protrusion in motile cells involves coordinated changes in the plasma membrane and the actin cytoskeleton. The long-standing question of whether growing actin filaments push the plasma membrane or they just stabilize the membrane deformation remains unresolved. Although the former idea dominated lately, recent data argue for the latter model by showing that the membrane-binding and -bending IRSp53/MIM domain (IMD) can induce actin-free filopodia-like protrusions. To understand how these filopodia generate and maintain their shape, we performed structural and kinetic analyses of IRSp53-IMD-induced filopodia in B16F1 melanoma cells by time-lapse microscopy and correlative electron microscopy. We found that F-actin-free IMD-induced filopodia were quite rare, and usually arose as retraction fibers. Accordingly, they did not display protrusive behavior, but were either stationary or retracting depending on their adhesion to the substratum. The majority of IMD-induced filopodia including all protruding filopodia, however, contained actin filaments, even though these filaments, as shown by EM, were less abundant, poorly bundled, and might not extend all the way to the filopodial tip. These results suggest that actin filaments are essential for protrusion. In protruding filopodia, the GFP-IMD and mCherry-actin labels often advanced in parallel, but sometimes mCherry-actin transiently lagged behind the GFP-IMD, suggesting that membrane deformation can precede the actin assembly. Interestingly, as revealed by EM of mildly extracted cells, IMD-induced filopodia displayed an unusual IMD-containing submembranous sheath encasing actin filaments, which might provide a structural support to actin-free filopodial segments. However, treatment with high concentrations of actin cytoskeleton-disrupting drugs eliminated the difference in the amount of filopodia-like extensions between control and IMD-expressing cells. Together, our data suggest that when the membrane and actin dynamics are uncoupled, actin filaments may not directly drive the protrusion, but only stabilize the space generated by the membrane deformation; yet, such stabilization is necessary for efficient protrusion. Supported by NIH grant GM 070898.

973/B181

Dynamin Regulates Apical Constriction in Epithelial Monolayers.

J. Chua, R. Rikhy, J. Lippincott-Schwartz; NICHD, CBMP, National Institutes of Health, Bethesda, MD

Apical constriction converts the shape of a polarized epithelial cell from a tall column into an inverted cone. This morphological process is requisite to the bending of epithelial monolayers observed during gastrulation, neural tube formation and the patterning of tubular organs such as the mammary gland, kidney and lung. Apical constriction is an effect of the acto-myosin contractile system remodeling at the apical junctional region. One molecule that has the ability to regulate actin is dynamin. This mechanoenzyme is known for its function in deforming membranes during endocytosis and for its dependence on GTP binding and hydrolysis to perform this function. Here, we use imaging approaches to probe dynamin's role in actin remodeling leading to apical constriction. We show that expression of a nucleotide free or GDP-bound form of dynamin (ie. Dyn2K44A), but not GTP-bound forms or other general endocytic inhibitors, leads to dramatic apical constriction of expressing cells in the MDCKII epithelial monolayer. This is dependent on dynamin's localization at the apical junctional region and on the activity of Rho-kinase (ROCK), but not of Myosin Light Chain Kinase (MLCK), to stimulate myosin IIB. A direct interaction of the nucleotide free/GDP-bound form of dynamin with cortactin is necessary to induce apical constriction, but this is independent of cortactin's interaction with Arp2/3 and N-WASP. Instead, cortactin's role may relate to it being a substrate for Erk kinase, as Erk kinase activity was necessary for apical constriction induced by Dyn2K44A expression. Our findings thus reveal that dynamin plays an important role in controlling acto-myosin-based apical constriction in polarized epithelial cells by orchestrating actin assembly and restructuring within these cells.

974/B182

Drosophila Cip4/Toca-1 Couples Actin Dynamics with Membrane Dynamics in the Wing Epithelium.

S. Bogdan, R. Fricke; Institut Fuer Neurobiologie, Münster, Germany

Developmental processes involving movement and cell shape changes are based on a highly dynamic reorganization of the actin cytoskeleton. Actin polymerization is controlled by members of WASP protein family, WASP, N-WASP and WAVE that activate the Arp2/3 complex. Biochemical work of a number of laboratories showed that a protein complex comprising Sra-1, Kette and Abi regulates WAVE function. We have recently shown that this complex also plays a central role in activating WASP mediated processes during Drosophila development. To understand how WAVE and WASP are differentially activated we have screened for additional regulatory factors. Here, we present a biochemical and functional analysis of a novel Abi interacting protein, the Cip4/Toca1 protein, a highly conserved member of the PCH-protein family. Interestingly, Cip4 can form protein complexes with both WASP and WAVE and is able to recruit WASP and WAVE in vivo to membrane tubules induced by Cip4. Loss and gain of function analysis reveal a so far unknown function of Cip4 for cytokinesis and the correct establishment of cell polarity during wing development, processes that require the coupling of actin dynamics and membrane trafficking. Moreover, biochemical and genetic data show that Cip4 is able to modulate not only WASP but also WAVE activity. Thus, Cip4 may define a new key component in directing cytoskeletal dynamics during membrane trafficking.

975/B183

Actin Polymerisation Traps Membrane Bound Proteins at the Leading Edge.

I. König, K. I. Anderson; Beatson Institute for Cancer Research, Glasgow, United Kingdom

Actin polymerisation is a highly dynamic process which drives many cellular events, including endocytosis and cell motility. It is known that actin monomers are added to the filaments at the leading edge of a migrating cell and that this polymerisation is the driving force of protrusion. Weisswange et al (1) found that the leading edge of fish keratocytes functions as a diffusion barrier for lipid dyes. The aim of the here presented study was to investigate weather this diffusion barrier is present in other cell types and if it is a general feature of protrusion. Fluorescence Recovery After Photobleaching (FRAP) was used as a method to measure the diffusion constant (D in µm2/s) of membrane anchored GFP (GFP-F) or free GFP (cytoplasmic). By comparing the rate of diffusion at protruding leading edges with the diffusion inside the lamellipodium we could show that the diffusion of GFP-F is significantly inhibited at the leading edge (D=3.89µm2/s) compared to lamellar regions (D=3.26 µm2/s). No reduction in diffusion could be observed at non protruding sites of the lamellipodial periphery (D=3.8 µm2/s), showing that the diffusion barrier depends on active protrusion. The diffusion of cytoplasmic GFP was not altered near the leading edge (D=18.36 µm2/s) compared to in the lamellipodium (18.2 µm2/s), indicating that only membrane bound proteins are affected. We suggest that this lipid diffusion barrier, previously only seen in fish keratocytes, is a general feature of protrusion. We propose that it traps regulatory proteins at the leading edge and can therefore be seen as a positive feedback mechanism for actin polymerisation. 1) Weisswange, I., Bretschneider, T., Anderson, K.I., 2005. The leading edge is a lipid diffusion barrier. J. cell Sci. 118, 4375-4380

976/B184

The Role of the Actin Cytoskeleton in the Loss of Membrane and Cell Wall Integrity during Inositolless Death in Saccharomyces.

B. Hanson, A. Calabretta; Biology, Canisius College, Buffalo, NY

When an inositol-requiring mutant of *Saccharomyces cerevisiae* (*MC6A*) is starved for inositol, there is a rapid loss of viability known as inositolless death and cell membranes disintegrate because of the depletion of phosphatidylinositol, a major membrane phospholipid. The loss of the actin cytoskeleton during inositolless death also contributes the disintegration of the membrane because of the loss of the three major F-actin structures: actin cables, actin-myosin contractile rings, and actin cortical patches. These structures play roles in the localization and synthesis of new cell wall and membrane in yeast. To measure weakening of the cell wall and membrane deterioration, the amount of protein in the medium released by the inositol-requiring mutant was measured following the initiation of inositolless death using the procedure of Hanson (J. Bact. 1980:143,18) Disintegration of the membrane following inositolless death leads to leakage of the yeast cytoplasm into the medium. Paralleling the decline in actin there was a 2-fold increase in protein (mg of protein released/ml medium) within 3 hours when compared to the control. By 24 hours there was a 6-fold increase in protein (mg of protein released/ml medium). As a measure of cell wall integrity, changes in amount of chitin and its localization were measured by staining cells with Calcofluor White M2R using the procedure of Pringle (Methods Enzymol 1991:194,729). Paralleling the decline in actin there was a 40% drop in the average cell chitin fluorescent intensity within 3 hours. By 24 hours the intensity was reduced 60%. The losses in the cell wall and membrane integrity during inositolless death can be attributed to the loss of the actin cytoskeleton which plays a role in the localization and synthesis of new cell wall and membrane in yeast, as well as the decline of inositol-containing phospholipids in the yeast cell membrane.

977/B185

Cell Spreading Dynamics: Actin Cortex Fluctuations and Plasma Membrane De-mixing.

D. M. Heinrich[1], G. Gerisch[2]; [1]Physics Department, Ludwig-Maximilians-Universitaet Muenchen, Munich, Germany, [2]Max-Planck-Institute for Biochemistry, Martinsried, Germany

Spreading of motile cells on solid surfaces is governed mainly by two factors, actin-network reorganization and membrane adhesion dynamics. We identify the interplay of both: We investigate spreading cells with an intact actin cortex on sub-second time scales, finding strong spatial and temporal fluctuations of actin turnover dynamics and therefore local gain and loss of cell-contact-area, leading to non-monotonous adhesion behavior. We used dual fluorescence labeling of filamentous actin and associated proteins, finding transient front-to-tail polarity. The fluctuating, discontinuous forces of protrusion and retraction in time and space prevent Dictyostelium cells from approaching a steady-state of adhesion, enabling a quick and locally defined reaction to external stimuli. Dictyostelium cells exhibit no stress fibers or pronounced focal adhesions. After de-polymerization of actin, cell spreading is governed by the lipid membrane self organization, which is enabled by the lack of membrane anchorage to the actin cortex. Cell stability is maintained by the microtubule network as well as the plasma membrane, with the membrane area and cell volume kept nearly constant. These cells continue to migrate, phagocytose and divide after repolymerization of the actin cortex. So, the living cell's spreading process without F-actin is governed by lipid-membrane self organization, leading to membrane de-mixing and to the formation of lipid domains, budding off into tubular membrane extrusions prior to substrate adhesion. These tubes form pearl-like structures with an average pearl diameter of 1µm. Initial cell adhesion is mediated by these pearl-like structures. Further spreading occurs by additional pearl-formation and their adhesion to the surface. Upon membrane staining with

labeled Concanavalin A (ConA), we observed the pearls to be ConA rich, whereas the plasma membrane, spanning the cell body, to lack the ConA label completely. The membrane composition is clearly very different in the adhesive pearls. The observed pearling morphology of the living cell membrane in the absence of F-actin indicates that the cell actively controls membrane morphology during adhesion and spreading via the actin cortex.

978/B186

A Step toward the Reconstitution of a Cell Cortex.

L. Pontani[1], J. Van der Gucht[2], C. Sykes[1]; [1]UMR 168, Institut Curie, Paris, France, [2]Laboratory of Physical Chemistry and Colloid Science, Wageningen, Netherlands

The composite and versatile structure of the cytoskeleton confers extremely complex mechanical properties to cells. In particular, actin assembly at the plasma membrane is the driving force for cell deformation and movement, while an actin cortex in conjunction with myosin motors provides sufficient contractility to generate shape changes of the cell. Simplified systems are useful tools for understanding the role of particular cytoskeleton structures in cell mechanics. For that reason we develop the following biomimetic approach for a better understanding of cell cortex dynamics. The necessary ingredients for actin polymerization are introduced into a liposome. Polymerization occurs at the membrane thanks to the grafting of polymerization activators on the inside layer of the liposome. Polymerisation is then triggered inside the liposome and the growth of actin cortex is successfully observed at the membrane. This experimental system paves the way to a thorough characterization of cortical dynamics under well-controlled conditions.

979/B187

Role of Cortical Rigidity in Spindle Positioning in C. elegans.

S. Redemann, J. Pecreaux, A. A. Hyman, J. Howard; Max Planck Institute for Molecular Cell Biology and Genetics, Dresden, Germany

The first cell division in C. elegans is asymmetric. Asymmetric cell division requires correct positioning of the mitotic spindle. Prior to metaphase, the nuclear-centrosome complex, the precursor of the mitotic spindle, is positioned in the cell center. During anaphase, the spindle is displaced towards the posterior so that bisection of the spindle during cytokinesis leads to daughter cells of unequal sizes. Forces that center and position the spindle come from cortical force generators that pull on astral microtubules. In order to generate force, the cortex needs to provide a stiff anchoring platform. However, a role for the cortex in C. elegans has only been described with respect to polarity establishment; its function as a provider of rigidity has not been reported. We perturbed the acto-myosin cortex by RNAi of non-muscle myosin II (nmy-2) using conditions that allowed us to separate polarity phenotypes from other roles of NMY-2. A striking phenotype of nmy-2 (RNAi) is the formation of invaginations: membrane tubes that are pulled from the plasma membrane into the cell. We visualized invaginations using labeled cortical and membrane-associated proteins (PAR-2 and phospholipase D-subunit). They were seen after RNAi against other actin cytoskeleton proteins including RHO-GEF, ROCK, ARP-2 and PFN-1. GPR-1/2, LIN-5, and G-alpha RNAi reduced invaginations, suggesting that the cortical force generators pull the invaginations. The formation of invaginations per se suggests a weakening of the cortex. As expected, we observed an increase in the variance of spindle position and orientation in nmy-2(RNAi). We used the oscillations of the centrosomes during anaphase as a reporter of spindle mechanics, and measured an increase in oscillations frequency but only a marginal decrease in amplitude. In order to understand this phenotype, we used our previously published model to analyze the results. Only by including the cortex into the model, we were able to fully describe the role of NMY-2. In summary, the occurrence of tubes after nmy-2(RNAi) strongly points towards a weakening of the cortex and the analysis of the spindle positioning suggests that the cortex provides a rigid platform for anchoring the force generators.

980/B188

Myrip Uses Distinct Domains in the Cellular Activation of Myosin Va and Myosin Viia.

J. S. Ramalho[1], V. S. Lopes[2], A. K. Tarafder[2], M. Seabra[2], A. Hume[2]; [1]1Centre of Ophthalmology, University of Coimbra, Coimbra, Portugal, [2]Molecular Medicine, Imperial College London, London, United Kingdom

Rab GTPases regulate organelle movement by recruiting effector proteins to membranes. Rab27a may regulate melanosome transport by switching melanosomes from microtubule- (MT-) to actin-mediated transport in retinal pigment epithelium (RPE) and skin melanocytes by recruiting related effector complexes; Myrip and myosin VIIa (MyoVIIa) and Melanophilin (Mlph) and myosin Va (MyoVa), respectively. Thus melanosomes are retained in RPE apical processes and melanocyte dendrites by linkage to actin. Myrip may also interact with MyoVa. To define the molecular basis of Myrip action we introduced Myrip and mutant variants into Myrip knockdown RPE cells and Mlph null melanocytes and assayed restoration of normal melanosome transport, a measure of MyoVIIa and MyoVa activity, respectively. We found that Myrip compensates loss of Mlph in melanocytes by restoring MyoVa-dependent melanosome transport. Mutational analysis reveals that Myrip-Mlph conserved domains are essential for this activity. Conversely, Myrip-specific inserts are essential for restoration of MyoVIIa-dependent melanosome transport in Myrip knockdown RPE. This suggests that Myrip-Mlph conserved domains and Myrip-specific inserts mediate cellular activation of MyoVa and MyoVIIa, respectively. We conclude that Myrip and Mlph comprise a family of Rab-associated myosin activating proteins where Myrip is a versatile activator of different myosins and Mlph is a specialized variant allowing MyoVa-dependent transport.

981/B189

Investigating the Role of Microtubule Dynamics in Asymmetric Spindle Positioning in the One-Cell C. elegans Embryo.

A. Werts, B. Goldstein; Biology, University of North Carolina, Chapel Hill, NC

Asymmetric cell divisions can result in daughter cells of unequal size. We are using the first division of the C. elegans embryo as a model to study how microtubule dynamics may contribute to the forces that asymmetrically position a mitotic spindle. The one-cell C. elegans embryo provides a valuable system to study this because one can combine genetics and high resolution live imaging of dynamic cytoskeletal movements during an asymmetric division. As the one-cell embryo undergoes its first mitosis, forces exerted on the microtubules position the mitotic spindle toward the posterior of the embryo, which leads to an asymmetric division plane. Some key modulators that have been

implicated in the initiation and regulation of these asymmetrically distributed forces include cortically localized PAR proteins, receptor independent G-protein signaling, and the microtubule motor protein dynein. While many of the players involved in asymmetric spindle positioning have been identified, the mechanisms of force generation are not completely understood. Labbé et al. 2003 and Kozlowski et al. 2007 have previously shown that microtubule plus-end residence times at the cortex are longer on the anterior half of the one-cell embryo compared to the posterior half. These residence times become symmetric in both halves of the embryo in polarity-defective and spindle positioning-defective backgrounds. From this evidence, it has been hypothesized that differential regulation of microtubule stability at the cortex might play a role in asymmetric spindle positioning. Currently, I am developing new techniques to image and analyze microtubule plus end dynamics at the cell cortex to better examine the importance of the microtubule tips and cortical proteins in spindle positioning. Also, by creating fiduciary marks on microtubules, I aim to resolve microtubule dynamics along single astral microtubules and study how these dynamics aid in force generation.

982/B190

Transient Interactions among Fission Yeast Proteins Tea1, Tea3 and Mod5 Regulate Protein Localization and Cell Polarity.

C. Bicho, H. A. Snaith, K. E. Sawin; Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, United Kingdom

Tea1 is a key regulator of cell polarity in S. pombe and is delivered from the cytosol to the cell tips by association with growing microtubule plus-ends. Once microtubules contact cell tips, Tea1 anchors to the cell membrane by a mechanism involving binding to Mod5, a membrane associated protein. Once at cell tips, Tea1 organises a polarised actin network by interacting with its downstream effectors. In previous work we showed that Tea1 and Mod5 regulate each other's localization at cell tips, through a proposed positive-feedback loop. In order to better understand the molecular mechanisms maintaining this regulation, we analysed Mod5 and Tea1 dynamics, using FRAP (Fluorescence Recovery After Photobleaching). We observed that tip-localised Mod5 is surprisingly mobile within the cell tip, while Tea1 turns over much more slowly. This suggests that transient interactions between Tea1 and Mod5 are responsible for maintaining Mod5 at cell tips via positive-feedback. Tea1 and Mod5 also bind to a third protein, Tea3, in the cell membrane. The three proteins can all bind to each other in a pair-wise fashion. When we over-expressed one of the three proteins we observed an increased interaction between the other two. Based on these results we propose that Tea1, Tea3 and Mod5 likely form a three-way complex at the cell tip. We created a Mod5 mutant in which Mod5-Tea3 interaction is disrupted but Mod5-Tea1 and Tea1-Tea3 interactions are normal. This mutant has polarity defects, even though Tea1 is anchored at the cell tips. This observation suggests that Tea1-anchoring and Tea1-recruitment of polarity machinery can be uncoupled, with the latter depending on the formation of what is likely a transient three-way complex of Tea1, Tea3 and Mod5 at cell tips.

983/B191

Domain Analysis to Dissect the Role of the Integrin Effector PINCH in Drosophila.

M. Elias[1,3], J. Kadrmas[1,3], M. Beckerle[1,2,3]; [1]Oncological Sciences, University of Utah, Salt Lake City, UT, [2]Biology, University of Utah, Salt Lake City, UT, [3]Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

Cell adhesion is required for morphogenesis and tissue integrity. Genetic evidence revealed a role for PINCH, a 5 LIM domain molecular scaffold, in integrin mediated adhesion at the cell membrane. In Drosophila, there are two well characterized binding partners for PINCH. Integrin Linked Kinase (ILK) binds LIM1 of PINCH, providing a link to integrins and to the actin cytoskeleton. ILK and PINCH zygotic mutants display embryonic lethality associated with disruption of actin-membrane interactions and resulting muscle failure. Ras Supressor-1 (RSU-1) binds to LIM5 of PINCH, stabilizes PINCH protein levels, and negatively regulates the JNK cascade. RSU-1 mutants are viable, but display wing blisters indicating a defect in integrin mediated adhesion. To further understand the specific functional contributions of PINCH binding to ILK and RSU-1, we have generated flies incorporating a transgenic PINCH (fused to GFP) with mutations that disrupt the interaction specifically with ILK (PINCH-Q38A) or RSU-1 (PINCH-D303V). The PINCH-Q38A transgene is able to fully rescue the PINCH null phenotype. This is surprising given the proposed role of ILK in the coordinated linkage of PINCH to integrin and actin. Furthermore, in the lateral epidermis and the dorsal vessel, PINCH-Q38A is mislocalized and displays a predominantly nuclear localization. These data suggest a possible nuclear role for PINCH and implicate ILK in the cytoplasmic sequestration of PINCH. The PINCH-D303V transgene only partially rescues the PINCH null phenotype and rescued flies display more severe wing blisters than RSU-1 mutants. These results raise the possibility that the D303V mutation impacts some other PINCH function in addition to RSU-1 binding. PINCH-D303V localizes properly to muscle attachment sites, but RSU-1 is absent from these sites, illustrating an essential role for PINCH in the recruitment of RSU-1 in the muscle. Taken together, these data indicate that neither PINCH binding to ILK nor to RSU-1 is sufficient to explain its essential role in viability and suggests the need for additional studies regarding the mechanism of action of PINCH.

984/B192

The Characterization of a Novel Membrane-Microtubule Linker Protein, CLIPR76.

J. Dzurisin, G. Ugrinova, H. Goodson; Chemistry and Biochemistry, University of Notre Dame, Notre Dame, IN

The microtubule cytoskeleton plays critical roles in cell structure and intracellular transport. Cytoplasmic linker proteins (CLIPs) help to link microtubules with membrane bound organelles. We have undertaken a study of genes related to CLIP-170 (the founding CLIP) in hopes of expanding our knowledge of microtubule-membrane interactions. This presentation will focus on one of these, CLIPR76. The CLIPR76 transcript contains three C-terminal CAP-Gly motifs (instead of the usual one or two) and has ankyrin repeats at the N-terminus. When transiently expressed in tissue culture cells, CLIPR76 localizes to and bundles microtubules. However, the CLIPR76 gene is also alternatively spliced, producing at least three additional transcripts in humans. The smallest of these, CLIPR76-4, encodes a 37kD protein containing only one CAP-Gly motif followed by a unique hydrophobic C-terminus. This protein localizes to and reorganizes the ER when expressed at high levels, and the hydrophobic C-terminus is necessary and sufficient to target the protein to the ER. CLIPR76-4 is homologous to another CLIPR protein, CLIPR59. The hydrophobic C-terminal region of CLIPR59 mediates localization of this protein to Golgi and the critical residues responsible for the localization have been identified (Lallemand-Breitenbach et al, 2004). It's intriguing that these proteins have similar hydrophobic tail regions that can specify localization to different organelles. Currently, we are investigating the C-terminal region of CLIPR76-4 in order to determine which amino acids of the hydrophobic tail are critical for the localization of CLIPR76-

4 to the ER membrane. While CLIPR59 is expressed predominantly in brain, CLIPR76-4 is highly expressed in muscle and its expression level dramatically increases as muscle cells develop. siRNA knockdown and fluorescence microscopy techniques are currently being utilized in order to determine what role CLIPR76-4 plays in muscle development.

985/B193

Charaterization of Two Distinct Membrane-Associated Pools of Adenomatous Polyposis Coli in Endothelial Cells.

E. S. Harris, W. James Nelson; Department of Biology, Stanford University, Stanford, CA

Adenomatous polyposis coli (APC) is a tumor suppressor commonly mutated in several cancers. APC is important for many fundamental processes including cell migration, cell adhesion, chromosome segregation, and transcriptional regulation. This large, multi-domain protein has many binding partners, likely contributing to its functional diversity. Although the importance of APC in diverse morphological processes is well established, little is known about the mechanism(s) by which APC and its binding partners coordinate these different cellular functions. We are using Human Umbilical Vein Endothelial Cells (HUVEC) as a model system to understand the cellular role(s) of APC and APC binding proteins. We find endogenous APC localizes to two sub-cellular regions in HUVEC: 1) in punctate clusters at the plus-end of microtubules that project into the extending cortex at membrane protrusions, and 2) at the lateral membrane, specific to sites of cell:cell contacts. Our results suggest that these two pools of APC maybe functionally distinct. Using drugs that disrupt either the microtubule or actin cytoskeleton, we find the localization of APC clusters in membrane extensions is dependent upon microtubules, while the lateral membrane localization of APC requires an intact actin cytoskeleton. In addition, the sub-cellular localization of APC binding proteins at each site is distinct. APC clusters partially co-localize with the microtubule plus-end tracking protein EB1, while APC at the lateral membrane exhibits robust co-localization with components of the adhesion complex, including beta-catenin, alpha-catenin, and VE-cadherin. Significantly, depletion of beta-catenin has no effect on APC clusters in membrane extensions but abolishes APC lateral membrane localization. These data suggest that localization of APC these different sub-cellular locations occurs through distinct binding partners and mechanisms. Future studies are aimed at determining the cellular roles of these two pools of APC.

986/B194

Ankyrin 2-Binding Activity Is Required for Spectrin Function in Drosophila, But Spectrin Still Assembles Upstream Of Ankyrin.

G. Mazock, A. Das, C. Base, R. Dubreuil; Laboratory for Molecular Biology, University of Illnois at Chicago, Chicago, IL

Spectrin and ankyrin are components of a protein scaffold that contributes to the structure and composition of the plasma membrane in animal cells. Recent studies of spectrin and ankyrin assembly in vertebrates and invertebrates found unexpected differences in their behavior. Vertebrate ankyrin functions upstream of spectrin, as gauged by the requirement of ankyrin for spectrin targeting. The opposite result was obtained in Drosophila where spectrin assembles independently of ankyrin 1 and is required to target ankyrin 1 to the plasma membrane. Here we asked if the same relationship held true for the recently described ankyrin 2 and spectrin. A recombinant Ankyrin-2-EGFP transgene was expressed in a variety of cells from wild type and β spectrin mutant embryos and larvae. The ankyrin-binding site spans two structural repeats in the β spectrin subunit (14 & 15). The relative contributions of each spectrin repeat were assessed using recombinant β spectrin transgenes in which each repeat was individually replaced with an irrelevant repeat from α spectrin. The results indicate that 1) ankyrin-2-EGFP behaves similar to ankyrin-1 in a wild type background; 2) modification of repeat 14 or 15 knocks out ankyrin-1 binding activity; 3) in contrast, ankyrin-2 binding activity of spectrin was primarily through repeat 14; 4) The repeat 14 mutant of β spectrin was a stable protein and was correctly targeted to the plasma membrane of polarized cells. However, unlike the repeat 15 mutant, the repeat 14 mutant altogether lacked β spectrin function in mutant rescue crosses. Thus, while ankyrin-1 binding activity was partially dispensable for β spectrin function, there was an absolute requirement for ankyrin-2 binding activity. The results establish that the essential function of spectrin is dependent on its interaction with ankyrin 2. Since ankyrin-2 is only believed to be expressed in the nervous system, it appears likely that spectrin has an essential function there too. Recent evidence from other labs indicates that ankyrin-2 modulates the behavior of the microtubule cytoskeleton in neurons. We suggest that spectrin functions to attach the ankyrin-microtubule complex to the plasma membrane.

987/B195

Ankyrin-B Is a Membrane Adaptor For Dynactin-4 at Skeletal Muscle Costameres.

G. Ayalon, J. Q. Davis, V. Bennett; Cell Biology and Howard Hughes Medical Insitute, Duke University Medical Center, Durham, NC

The dynactin complex is required for transport of diverse organelles along microtubules. Dynactin-4, which is located at the pointed-end of the dynactin Arp1 filament, has been proposed to couple dynactin to membrane cargo. We present evidence that ankyrin-B is a membrane adaptor for dynactin-4 in skeletal muscle and couples dynactin-4 to costameres. Costameres are specialized membrane domains where force is transmitted from the contractile apparatus to the extracellular matrix. Costameres contain dystrophin, which binds to actin, as well as the transmembrane protein beta-dystroglycan which links dystrophin to laminin. We have found that the adaptor protein ankyrin-B binds to dystrophin and also is associated with costameres. Moreover, a Becker muscular dystrophy mutation that reduces ankyrin-B-binding also impairs sarcolemmal localization of dystrophin-Dp71. We identified dynactin-4 as a potential ankyrin-B-binding protein in a yeast two-hybrid screen. Ankyrin-B binds to dynactin-4 in solution with a Kd of 50 nM. Dynactin-4 is localized in skeletal muscle in micron-sized puncta that align with costameres and costamere-associated ankyrin-B. A population of microtubules also is associated with costameres. In vivo knockdown of ankyrin-B by siRNA in adult mouse skeletal muscle results in loss of costamere-associated dynactin-4. In addition, the costamere-associated microtubules also disappear. Dystrophin and beta-dystroglycan remain intracellular in ankyrin-B-depleted muscle, and beta-dystroglycan accumulates in a compartment close to the trans-Golgi network. These results demonstrate that ankyrin-B is a molecular partner for dynactin-4 in skeletal muscle and is required for association of dynactin-4 with costameres. Moreover, these findings suggest dual roles for ankyrin-B in delivery of dystrophin to the sarcolemma: first in stabilizing costamere-associated microtubules through dynactin-4 and the dynactin complex, and second in coordinating transport of newly synthesized dystrophin and dystrophin-associated proteins along these costamere-associated microtubules through direct interaction with dystrophin.

988/B196

α-2 Giardin, of the Annexin Gene Family, Dynamically Localizes to the Ventral Disc and Contributes to Attachment in Giardia intestinalis.

S. House, J. Pham, S. C. Dawson; UC Davis, Davis, CA

Giardia intestinalis is a widespread intestinal parasite with a complex cytoskeleton (ventral disc, median body, and eight flagella) that plays a crucial role in its virulence. Though ventral disc dynamics are proposed to be necessary for attachment to the intestinal microvilli, little is known of this process at the molecular level. α-Giardins, of the annexin gene family, are a diverse and highly conserved multi-gene family of Ca2+-dependent, phospholipid-binding proteins. Previous studies have shown that several localize to the ventral disc. Though their physiological function remains largely unknown, we propose that annexins link the plasma membrane to the cytoskeletal components of the disc and modulate the proteins responsible for host attachment. To determine its role in attachment, we first GFP-tagged and localized α-2 giardin in fixed and live cells. To test the function of α-2 giardin in flagellar beating and attachment, we created inducible dominant negative α-2 giardin mutants at highly conserved calcium binding sites. We used live imaging with fluorescent and DIC (differential interference contrast) microscopy to characterize single and double calcium-binding mutants. mRNA expression for mutant strains was measured relative to actin, and the non-mutagenized GFP-tagged strain by QPCR. α-2 giardin::GFP primarily localizes to the ventral flagella and the ventral disc. Analysis of α-2 giardin turnover using FRAP (fluorescent recovery after photobleaching) shows that α-2 giardin is highly dynamic, recovering within seconds of photobleaching. Early data suggests that inhibition of calcium-binding sites in α-2 giardin impairs the sigmoidal beat of the ventral flagella. Lastly, we observe defects in the structure of the ventral disc plasma membrane in the dominant negative mutants, implying that α-2 giardin could contribute to attachment dynamics.

989/B197

Antagonistic Expression of the ERM Proteins Ezrin and Moesin during Initial Stages of Epithelial to Mesenchymal Transition.

J. Srivastava, N. Madson, D. Barber; UCSF, San Francisco, CA

Cancers of epithelial origin are proposed to undergo an epithelial to mesenchymal transition (EMT) to become metastatic, by developing a fibroblast-like phenotype with enhanced migratory capacity. A hallmark feature of EMT is remodeling of the membrane-cytoskeleton architecture to promote epithelial plasticity. We found that during EMT of NMuMG mammary epithelial cells treated with TGF-beta there is antagonistic expression of the ERM proteins ezrin and moesin, which link the actin cytoskeleton to the plasma membrane. During initial stages of EMT (1-3 d) ezrin expression decreases ~ 70 % and moesin expression increases ~ 200%. The decrease in ezrin during initial stages of EMT is in marked contrast with an established increase in ezrin expression in many metastatic carcinomas, and we found that in later stages of EMT (6 to 7 d) with continued treatment of TGF-beta, ezrin expression increases. This biphasic expression of ezrin with an initial decrease followed by an increase, is also in marked contrast to with the signature of EMT repressors or inducers that exhibit a single phase of expression or activity. Preliminary studies suggest that a decrease in ezrin may be necessary for initiating EMT. Transient overexpression of ezrin in NMuMG cells had no effect on cell morphology in the absence of TGF-beta, but suppressed loss of cell-cell contacts and translocation of E-cadherin from the plasma membrane to the to cytosol. We predict a previously unrecognized biphasic expression of ezrin may be necessary for cancer progression. Initial suppression of ezrin expression in epithelial cells might allow initiation of EMT by promoting epithelial plasticity through a loss of apical microvillus architecture and intercellular adhesions. A subsequent increase in ezrin expression in differentiated mesenchymal cells maintains cell shape and promotes migratory behavior.

990/B198

LOK Is the Dominant ERM Kinase in Resting Lymphocytes and Regulates Cytoskeletal Rearrangement through ERM Phosphorylation.

N. Belkina, Y. Liu, J. Hao, S. Shaw; EIB, NIH/NCI, Bethesda, MD

Proteins of the ERM (Ezrin-Radixin-Moesin) family mediate critical linkage of actin cytoskeleton to plasma membrane in many cells, including lymphocytes. ERM activity is regulated in part by phosphorylation at a C-terminal Threonine, but compelling confirmation is lacking for any of the proposed mammalian ERM kinases. Studies herein demonstrate that a previously obscure kinase, Lymphocyte-Oriented Kinase (LOK) is an efficient ERM kinase in vitro, and the dominant ERM kinase in lymphocytes. Mass spectrometric analysis and immunofluorescence studies demonstrate that LOK is appropriately localized at the plasma membrane to mediate ERM phosphorylation. Peptide specificity analysis shows it to be a basophilic kinase with a predicted optimum sequence resembling the ERM site, including an unusual preference for tYrosine at P-2. Protein phosphorylation analysis demonstrates preferential phosphorylation of ERM compared to two favored candidate ERM kinases: PKC and ROCK. Two genetic approaches demonstrate a role for LOK in ERM phosphorylation: Jurkat cell transfection with LOK kinase domain augments ERM phosphorylation and lymphocytes from LOK knockout mice have >50% reduction in ERM phosphorylation. Taken together the findings on localization and specificity argue strongly that LOK is a direct ERM kinase. The knockout mice have normal hematopoietic cell development but notably their migration and polarization in response to chemokine are enhanced. These functional alterations make sense in the context of current understanding of the role of ERM phosphorylation in regulating cortical reorganization. Thus, these studies identify a new ERM kinase of major importance in lymphocytes and elucidate a role for it in regulating cell shape and motility.

991/B199

Dual Role of the Exocyst in Exocytosis and Actin Reorganization during Invadopodia Formation.

J. Liu[1], S. Mueller[2], W. Guo[1]; [1]Biology, University of Pennsylvania, Philadelphia, PA, [2]Oncology, Lombardi Comprehensive Cancer Center, Georgetown University Medical School, Washington, DC

Invadopodia are actin-rich membrane protrusions formed by tumor cells that degrade the extracellular matrix for invasion. Invadopodia formation involves membrane protrusions driven by Arp2/3-mediated actin polymerization and secretion of matrix metalloproteinases (MMPs) at the focal degrading sites. The exocyst mediates the tethering of post-Golgi secretory vesicles at the plasma membrane for exocytosis. Recently, the exocyst has also been implicated in cell migration. Here, we report that the exocyst plays a pivotal role in

invadopodial activity. With RNAi knockdown of the exocyst components Exo70 and Sec8, MDA-MB-231 cells expressing mutant Y527F c-Src failed to form invadopodia. Furthermore, the secretion of MMP-2 and MMP-9 was inhibited. On the other hand, overexpression of Exo70 promoted invadopodia formation. Immunofluorescence microscopy localized Exo70 and Sec8 at the focal degrading sites. We have also found that the exocyst interacts with the Arp2/3 complex in cells with high invasion potential; blocking the exocyst-Arp2/3 interaction inhibited invadopodia formation. Together, our results suggest that the exocyst plays a dual role in cell invasion by mediating the secretion of MMPs at focal degrading sites and regulating Arp2/3-mediated actin reorganization.

992/B200

Protein 4.1R Regulates Cell Motility by Modulating Beta-1 Integrin Mediated Signal Transduction in Mouse Embryonic Fibroblast.

L. Chen, Q. Kang, N. Mohandas, X. An; Red Cell Physiology Lab, New York Blood Center, New York, NY

Protein 4.1R (4.1R) is the prototypical member of protein 4.1 superfamily including 4.1 family, ERM family and talin. 4.1R was first discovered in red cell where its role in linking transmembrane proteins to the cytoskeleton has been well established. Recent evidence suggests that 4.1R is widely expressed in a variety of cells such as epithelial cells, T cells, B cells and mast cells. However the role of 4.1R in other cells remains largely unexplored. Here we investigate the role of 4.1R in fibroblasts using embryonic fibroblasts derived from wild type and 4.1R knockout mice. We show that 4.1R-null embryonic fibroblasts display impaired cell spreading, cell migration and cell motility. We further show that expression and activation of beta-1 integrin are significantly reduced in 4.1R-null fibroblasts. Moreover, fibronectin-induced phosphorylation of focal adhesion kinase, the immediate downstream molecule of beta-1 is also impaired. Our findings demonstrate that 4.1R is required for the assembly and activation of beta-1 integrin and that diminished activity of beta-1 integrin leads to decreased intracellular signal transduction which in turn affects cell spreading, migration and motility of MEF cells.

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993/B201

Marshalin, a de novo Cytoskeleton-organizing Protein.

J. Zheng, K. K. Miller, S. Sengupta, M. Cheatham, P. Dallos; Communication Sciences and Disorders, The Hugh Knowles Center, Northwestern University, Evanston, IL

Cochlear inner and outer hair cells (OHCs) are mechanoreceptors capable of transforming sound waves into electrical signals. Each hair cell displays a staircase array of stereocilia. A “tip link” connects the top of each shorter stereocilium to the side of its taller neighbor and delivers force to the mechano-electric transducer channels upon deflection of the ciliary bundle. Cadherin 23 (CDH23) is one of two proteins that comprise the tip link. Identifying proteins associated with CDH23 could greatly enhance our understanding of cochlear function. Using the membrane-based yeast two-hybrid system to screen a newly built OHC-cDNA library, we identified a de novo protein, which we name marshalin, as a potential partner of CDH23. When mammalian cells were transfected with marshalin cDNA, three expression patterns were observed. Type I shows a typical protein expression pattern with marshalin staining dots spreading throughout the cytoplasm. Type II has short “sticks”, while type III appears as long “strings,” some of which are over 50um in length and expand to form spindle-shaped tubes. By using immunofluorescence, we examined the cytoskeleton and other membrane organelles in marshalin-expressing cells. Our data indicate that marshalin and tubulin staining co-localize, as does marshalin and actin. Although the Golgi network is disrupted, the Golgi marker protein Golgi97 co-localizes with marshalin. Mitochondria, however, are not affected by marshalin. In order to investigate the relationship between the cytoskeleton and the stick/string formations in marshalin-expressing cells, we applied colchicine to disrupt microtubules and latrunculin A to disrupt actin filaments. As a result, type II and III patterns disappeared with colchicine, but were still observed following latrunculin A treatment. However, even when most actin filaments were disrupted, actin staining continued to co-localize with marshalin-stick/string formations. These data suggest that marshalin is a cytoskeleton-organizing protein that marshals the entire cytoskeletal structure to form stereocilia-like hair structures inside cells. (Work supported by NIH Grants DC00089, DC006412 and The Hugh Knowles Center Leadership Fund).

994/B202

CTGF Activates the Canonical Wnt Pathway in Human Mesangial Cells.

B. Rooney, J. Crean, H. O Donovan, A. Gaffney, M. Browne, C. Godson; Conway Institute, University College Dublin, Dublin, Ireland

Diabetic Nephropathy (DN) is the leading cause of end-stage renal disease worldwide and a condition that affects 45% of type II diabetes patients. Diabetic glomerular fibrosis is caused by accumulation of extracellular matrix proteins in the mesangial interstitial space resulting in mesangial expansion and ultimately End Stage Renal Failure (ESRF). Recent efforts in our group have focused on therapeutic strategies based on modulators of TGF β and mediators of TGF β, including the prototypic CCN family member CTGF. Studies have begun to unravel how the functional domains within the CTGF modules orchestrate signals and control key biological processes. Here we describe the effects of CTGF on the canonical Wnt pathway in human mesangial cells. Our studies show that CTGF not only interacts with the Wnt pathway, but has the capacity to activate it. Treatment of primary human mesangial cells with CTGF leads to increased phosphorylation of GSK-3 β, resulting in accumulation and nuclear localisation of β catenin, both of which are hallmarks of canonical Wnt signaling. Results from promoter reporter assays show that CTGF causes an increase in TCF/LEF activity and downstream transcriptional activation of the canonical Wnt pathway. Furthermore, mutational studies have demonstrated that CTGF is capable of manipulating the Wnt pathway via its Von willebrand repeat containing N- terminal domain, likely through interaction with the Wnt co-receptor LRP 5/6. Our studies show that the N-terminal region can activate the Wnt pathway, bringing about nuclear localisation of β catenin and phosphorylation of GSK-3 β. Previous studies have shown that Wnt signaling can modulate cytoskeletal rearrangement and matrix protein accumulation and taken together, these findings suggest that CTGF/Wnt signaling may play a crucial role in the pathogenesis of DN.

995/B203

Actin Rearrangements in Disassembly of Cadherin-Based Cell-Cell Contacts during EMT.

N. H. Bishop, R. Sperry, M. Carter, D. Staley, M. Hansen; Physiology and Developmental Biology, Brigham Young University, Provo, UT

During normal development and in cancer metastasis, cell-cell adherent, polarized epithelial cells become individual, migratory cells—a process termed epithelial-mesenchymal transition or EMT. EMT requires radical reorganization of the actin cytoskeleton, including stress fiber remodeling and membrane protrusion formation, which contribute to increased cell motility. Myosin-based contractility is coordinated with increased motility to drive rupture of cell-cell junctions, an essential step in EMT. However, little is known about how actual actin rearrangement events might contribute to disassembly of cell-cell adhesions. Here we use time-lapse imaging and fluorescence microscopy to address this. Contacting, epithelial MDCK cells display tightly bundled actin filaments that run parallel and immediately juxtaposed to adherens junctions (AJs) and terminate at cell vertices. Scatter factor treatment causes dramatic remodeling of this actin structure. Actin fibers in bundles parallel to the AJ detach from each other and the cell-cell contact, their lengths exhibiting rearward movement and their ends remaining connected to junctions at cell vertices. Actin assembly briefly increases at vertices, forming a large patch of actin that quickly disaggregates into smaller puncta. These puncta move laterally away from vertices, carrying with them the actin fibers that have detached from the large actin bundle parallel to cell-cell contacts. Detachment is preceded by the loss of α-actinin from the junction, indicating that the function of actin bundling proteins in maintaining actin structure at cell-cell adhesions must be abrogated during EMT. Rupture of junctions begins between puncta, expanding until the puncta are the only remaining contact sites between cells. Puncta stain for cadherin and zyxin, indicating that they are specialized sites of actin-membrane linkage. Expression of a zyxin mutant alters detachment of cell-cell contacts during EMT, supporting this idea. These observations provide new detailed insight into actin rearrangements during disassembly of AJs and define actin regulatory systems that likely contribute to this process.

996/B204

CRMP1 - a Candidate Dystonin Interacting Protein.

K. Bhanot[1,2], K. Young[1,2], R. Kothary[1,2,3]; [1]Ottawa Health Research Institute, Ottawa, ON, Canada, [2]Department of Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada, [3]Department of Medicine, University of Ottawa, Ottawa, ON, Canada

Dystonin/Bpag1 is a large cytoskeletal protein that contributes to subcellular structure. Loss of dystonin in mice results in neuromuscular dysfunction and early death. This phenotype is caused by a sensory neuropathy which results in abnormal posturing, uncoordinated muscle movements and poor muscle tone. The large size of dystonin isoforms and varied subcellular localizations suggest that dystonin isoforms are likely to be involved in multiple protein interactions. Known interactions occur with actin filaments and microtubules, and we have previously demonstrated that dystonin can also interact with an outer nuclear envelope (NE) protein, nesprin-3alpha. We have performed pull-down assays in search of interacting proteins of the neuronal dystonin-a2 isoform using fusions of the plakin domain. The CRMP1 protein has been identified as a result of these efforts through mass spectrometry analysis. The CRMP family of proteins are essential for neuronal development by regulating cytoskeletal organization. Current efforts are directed towards validating the interaction between dystonin and CRMP1 through co-immunoprecipitation and co-immunofluoresence assays. Our work will help shed light on the functional role of the versatile dystonin protein and highlight its involvement in the cytoskeletal pathways of the cell. This will further facilitate the understanding of how cytoskeletal proteins affect and regulate various neurodegenerative disorders.

997/B205

βPix Regulates Integrin-Mediated Cell Spreading through Interaction with Dyn2.

S. Yang[1], S. Kang[1], S. Rhee[2], D. Park[1]; [1]School of Biological Sciences, Seoul National University, Seoul, South Korea, [2]Department of Life Science, Chung-Ang University, Seoul, South Korea

The p21-activated protein kinase (PAK) interacting exchange factor (βPix-a/p85COOL-1/p85SPR) is known as a guanine nucleotide exchange factor (GEF) for Rac and Cdc42. It has been reported that βPix is required for Rac-mediated actin reorganization at the membrane of migrating cells. Recently, βPix has been focused on the involvement in the integrin-dependent signaling processes at cell adhesion sites. In this study, we identified a large GTPase, dynamin as a novel βPix binding protein to the βPix SH3 domain. We found that the interaction between dynamin2 (Dyn2) and βPix increased βPix-mediated Rac activation. Then, we investigated whether the interaction between βPix and Dyn2 could affect spreading of cells. Overexpression of Dyn2 enhanced cell spreading on fibronectin, whereas overexpression of Dyn2 mutant, which hardly binds to βPix, did not. In addition, double knock-down of βPix and Dyn2 by siRNA caused suppression of cell spreading. Meanwhile, constitutively active Src kinase increased βPix-Dyn2 binding and PP2, Src kinase specific inhibitor, blocked the binding. These results suggest that Src kinase regulates interaction between βPix and Dyn2. Taken together, these findings demonstrate that Dyn2 functions as a positive regulator for βPix in Rac-mediated cell spreading, and provide a new insight that Src kinase may regulate integrin-dependent cell spreading through mediation of the interaction between βPix and Dyn2

998/B206

Analysis of γ-adducin Gene (Add3) Expression Using an EGFP Knockin Strategy in Mice.

K. Sahr, A. Lambert, S. Ciciotte, L. Peters; The Jackson Laboratory, Bar Harbor, ME

The adducins are a family of three closely related cytoskeletal proteins (α, β, and γ) encoded by separate genes. While the α- and γ-adducin genes are expressed in all tissues examined, β-adducin gene expression is more restricted with highest levels in erythroid cells and the brain. In red blood cells (RBCs), where it has been most studied, adducin in the form of heterodimers and heterotetramers is localized to the spectrin-actin junctional complex of the peripheral membrane skeleton. In order to study γ-adducin gene expression in greater detail we have made a targeted knockin of an enhanced green fluorescent protein (EGFP) reporter into the first coding exon of the mouse γ-adducin gene. EGFP expression was then analyzed in adult blood, spleen, and bone marrow tissues by flow cytometry, and in developing embryos by fluorescence imaging. Circulating RBCs were EGFP-, which is consistent with the low levels of γ-adducin protein detected in mature RBC membrane ghosts. In bone marrow cells of nontreated mice or in the spleens of mice made anemic by injection with phenylhydrazine where the rate of erythropoiesis is greatly elevated, all red cell progenitors identified by labeling with CD71 and Ter119 are EGFP-. Essentially all

circulating or splenic T cells, B cells, monocytes, and granulocytes displayed moderate levels of EGFP expression. While EGFP expression was detected at low to moderate levels in most tissues throughout embryonic development, high levels were observed in the forming heart beginning at approximately E8.5. EGFP expression was relatively uniform during formation of the heart tube and tube folding, and at the later stages of chamber formation and heart growth. Additional experiments are ongoing to analyze the expression of γ-adducin during embryonic development, especially heart formation, in more detail.

999/B207

A Novel Role for an APC2-Diaphanous Complex in Regulating Actin Organization in Drosophila.

R. L. Webb, M. Zhou, M. Kapur, B. McCartney; Biological Sciences, Carnegie Mellon University, Pittsburgh, PA

Organization and rearrangement of cytoskeletal elements is essential for many cellular processes. The tumor suppressor Adenomatous polyposis coli (APC) affects the function of both microtubules and actin, but the precise mechanisms by which it does so are not well understood. Drosophila syncytial embryos are an excellent in vivo model for understanding the role of APC proteins in organizing the cytoskeleton, as the coordination of both actin and microtubules is necessary for cytoskeletal rearrangements involved in synchronous nuclear divisions. We examined Drosophila syncytial embryos null for APC2 and observed defects in the formation and extension of pseudocleavage furrows, cortical actin structures important for mitotic fidelity in early embryos. Furthermore, we show that the formin Diaphanous (Dia) functions with APC2 in this process. Colocalization of APC2 and Dia peaks during actin pseudocleavage furrow extension, and localization of APC2 to furrows is Dia dependent. Furthermore, we have shown that APC2 binds Dia directly through a region not previously shown to interact with Dia-related formins. Consistent with these results, reduction of diaphanous enhances actin defects in APC2 mutant embryos. Thus, an APC2-Dia complex appears critical for actin furrow extension in the syncytial embryo. Interestingly, EB1, a microtubule +TIP and partner of vertebrate APC1 and mDia1, may not function with APC2 and Dia in furrow extension. Finally, while Dia-related formins are activated by Rho family GTPases, our data suggest that the APC2-Dia complex may be independent of Rho1. Together, these data suggest a model in which APC2 and Dia function in a novel complex to promote the extension of actin-based furrows in the Drosophila embryo.

1000/B208

The Role of the B Cell Antigen Receptor in Cytoskeletal Rearrangements.

S. Machtaler[1,2], C. L. Jang[1,2], M. Dang-Lawson[1,2], C. C. Naus[1,3], L. Matsuuchi[1,2]; [1]Life Sciences Institue, University of British Columbia, Vancouver, BC, Canada, [2]Cell Biology Group/Zoology, University of British Columbia, Vancouver, BC, Canada, [3]Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada

The B cell antigen receptor (BCR) is a membrane bound receptor on B-lymphocytes that binds specific antigens providing signals that regulate B cell development and survival. It is comprised of an antigen binding subunit and a signaling subunit (Igα/β) that activates downstream signaling pathways. The cytoskeleton is also a target of BCR signaling and its rearrangement mediates B cell adhesion, spreading, polarization and formation of an immunological synapse. BCR and chemokine signaling both contribute to promoting cell polarity, movement and migration. B cell spreading and actin-rich immune synapse formation were used as readouts to investigate whether the composition of the cytoplasmic domains of the BCR have effects on B cell shape, presumably through rearrangement of the cytoskeleton. Our results showed that when the BCR was mutated such that there were two Igβ tails or one Igβ tail, there was a reduction in signaling, B cell spreading and actin-rich synapse formation compared to that of the WT BCR. These results imply a differential requirement for the cytoplasmic domains of Igα/β in normal BCR mediated cytoskeletal rearrangements and highlights the importance of Igα. In addition we have tested if the gap junction intercellular communication protein, Connexin 43 (Cx43) has any effects on B lymphocyte spreading responses. This is because Cx43 is reported to influence cell migration in neurons and glial cells. We have found a correlation with Cx43 expression and the ability of lymphocytes to respond to BCR-induced cytoskeletal rearrangements. The interactions among these components are being addressed by mutational analysis. (This work was supported by grants from CIHR and NSERC to L.Matsuuchi)

1001/B209

Coordinated Activation of Protein Kinase C Isoforms in Podosome Formation and Function in Human Lung Bronchial Epithelial Cells.

H. Xiao[1, 2], X. Bai[1], A. Kapus[3], A. S. Mak[4], M. Liu[1, 2]; [1]Division of Cell and Molecular Biology, University Health Network Toronto General Research Institute, Toronto, ON, Canada, [2]Department of Physiology, Faculty of Medicine, University Of Toronto, Toronto, ON, Canada, [3]Department of Biochemistry, Faculty of Medicine, University of Toronto, Toronto, ON, Canada, [4]Department of Biochemistry and Protein Function Program, Queen's University, Kingston, ON, Canada

Podosome is a cellular structure that is responsible for cell migration and invasion by breaking through the extracellular matrix barriers. Previously we reported that PDBu, a PKC activator, induced the formation of podosome-like structures in primary normal human lung bronchial epithelial cells; however the role of each PKC isoform in PDBu-induced podosome formation and proteolytic capability is largely unknown. Herein, we demonstrated that PDBu treatment increased phosphorylation of PKC δ, θ, μ and ζ/λ, but not the phosphorylation of cPKC (α, βI or βII) in human bronchial epithelial BEAS2B cells. Using immunofluorescent staining, we found that all these PKC were translocated from cytosol to the sites of podosomes and membrane circular dorsal ruffles/waves upon PDBu treatment. BIM I and Ro-31-8220, pharmacological blockers of both cPKCs and nPKCs; Gö6976, a cPKC inhibitor; BAPTA/AM, an intracellular Ca2+ chelator to prevent the translocation of cPKCs, and PKC α siRNA, prevented PDBu-induced podosome assembly. Interestingly, although Rottlerin, an inhibitor for nPKC family, and siRNA specific for PKC δ, μ or ζ, did not affect formation of podosomes, they significantly reduced degradation of gelatin-coated extracellular matrix. siRNAs to individual PKC isoforms specifically reduced protein level of designated PKC isoform, also selectively reduced phosphorylation of other PKC isoforms, suggesting a cross-talk among PKCs. Rottlerin pretreatment had no effect on PDBu-induced translocation of PKCα to podosomes, but blocked translocation of PKC ζ. Furthermore, a pseudosubstrate peptide for PKC ζ blocked the accumulation and enzymatic activity of secreted MMP-9 in culture medium. In conclusion, our results demonstrate that PDBu-induced podosome formation is mainly mediated through cPKCs especially PKCα, which further recruit other PKC isoforms to

the podosomes. However, the proteolytic capability of podosomes mainly depends on aPKCs particularly PKC ζ, which may be recruited to podosomes via nPKCs, such as PKC δ. Thus, formation and proteolytic activity of podosomes are regulated by different PKC isoforms in a coordinated fashion.

1002/B210

Supervillin Couples Myosin-Dependent Contractility to Podosomes and Enables Their Turnover.

S. Cornfine[1], T. Smith[2], Z. Fang[2], E. Luna[2], S. Linder[1]; [1]Institute for Prevention of Cardiovascular Diseases, LMU, Munich, Germany, [2]Dept. of Cell Biology, Cell Dynamics Group, University of Massachusetts Medical School, Worcester, MA

Podosomes are actin-rich adhesion and invasion structures of monocytic, endothelial or smooth muscle cells. Particularly in macrophages, podosomes exist in two distinct subpopulations displaying different sizes and dynamics: large, so-called precursors at the cell periphery or the leading edge, which divide or dissolve within 2-12 minutes and "regular" podosomes in the inner regions of the substrate-attached part, which are smaller and more stable. We show here that the membrane-associated protein supervillin is mostly present at "regular" podosomes, but not at precursors. Supervillin becomes enriched at precursors during their dissolution, pointing to a potential role in podosome turnover analogous to supervillin-mediated disruption of focal adhesions (Takizawa et al., 2006, J. Cell Biol 174, 447). Indeed, overexpression of EGFP-supervillin decreases podosome numbers. Of the two supervillin sequences shown to play a role in focal adhesion turnover (aa 1-174 and aa 342-571), only SV1-174, which binds myosin IIA heavy chain and myosin light chain kinase, significantly affects podosome regulation in macrophages. A deletion construct (SV174-1792) i) does not decrease podosome numbers, ii) no longer differentially localizes to only "regular" podosomes, associating also with precursors, and iii) no longer binds myosin IIA although it retains actin-binding capacity. These results indicate that differential recruitment of supervillin to podosome subpopulations is dependent on actomyosin binding/contractility, with mutants devoid of myosin-binding activity acting as actin-binding proteins without affecting podosome turnover. Time-lapse analyses confirm that enrichment of EGFP-supervillin at podosomes coincides with coupling of these structures to myosin IIA-positive cables and subsequent podosome dissolution. In sum, we show that podosome subpopulations have different molecular compositions and identify supervillin as the first protein to be differentially recruited to these subpopulations. Supervillin appears to play a critical role during podosome dissolution by coupling myosin II-dependent contractility to podosomes.

1003/B211

Periostin, a Fasciclin I Domain Containing Protein, Interact with TGFBI and Accumulated with the Mutated TGFBI in Corneal Deposits of Granular Corneal Dystrophy Type II.

B. Kim[1,2], S. Choi[1], T. Kim[1,2], S. Ahn[1], E. Kim[1,2]; [1]Department of Ophthalmology, Corneal Dystrophy Research Institute, Yonsei Uinversity College of Medicine, Seoul, South Korea, [2]Department of Medical Engineering, Brain Korean 21 Projects Team of Nanobiometerials for Cell-based Implants, Yonsei University, College of Medicine, Seoul, South Korea

The 5q31-linked corneal dystrophies are heterogeneous autosomal dominant inherited eye disorders that are characterized by progressive accumulation of protein deposits in the cornea, resulting in severe vision impairment. The 5q31-linked corneal dystrophies are commonly caused by mutations in transforming growth factor-beta induced gene, TGFBI. However, despite the isolation of its disease responsible gene, the cellular roles of TGFBI and molecular mechanisms of mutated TGFBI accumulation process in cornea remain poorly understood. Here we report the identification of periostin, a highly TGFBI-related molecule, as a specific TGFBI binding partner. We have verified the physiological relevance of this interaction using in vivo and in vitro biochemical and immunofluorescence microscopic analyses. And we find that the association of TGFBI and periostin is mediated by N-terminal cysteine rich EMI domain contained regions of both TGFBI and periostin. 5q31-linked corneal dystrophies-associated mutations of TGFBI cause to affect the interaction with periostin in vivo and subcellular localization of TGFBI. Furthermore, we also shown here that periostin was co-stained and accumulated in the TGFBI positive deposit in the cornea of granular corneal dystrophy type II (also known as Avellino corneal dystrophy) patient. Our findings suggest that the functions of highly related two proteins, TGFBI and periostin, are in the same biochemical pathway and periostin plays an important roles for the pathogenic development of 5q31-linked corneal dystrophies.

1004/B212

Mutation in Sh3pxd2b Causes Congenital Glaucoma in Mice.

M. Mao[1], M. Anderson[1,2]; [1]Molecular Physiology and Biophysics, The University of Iowa, Iowa City, IA, [2]Ophthalmology and Visual Sciences, The University of Iowa, Iowa City, IA

Congenital forms of glaucoma (CG) refer to a spectrum of ocular diseases that cause glaucomatous optic nerve degeneration in newborns and the young. In humans, CG exhibits strong hereditary links but many of the underlying genetic factors remain unknown. nm2702 mice are a recently discovered mouse model of CG. Mice homozygous for the spontaneously arising nm2702 mutation have several developmental abnormalities including runted growth, craniofacial abnormalities, and malformation of multiple ocular structures. Histological analysis indicates a severe and rapid loss of optic nerve axons, a hallmark of glaucoma. The goal of this project was to identify and characterize the disease causing mutation in the nm2702 mouse model of CG and to expand the molecular pathways underlying CG. Using fine scale mapping and bioinformatics-driven sequencing strategies, we have identified the cause of the nm2702 phenotype as a 1 bp deletion within the Sh3pxd2b gene. Sh3pxd2b is a newly annotated gene about which little is known. SH3PXD2B contains one PX domain and four SH3 domains. The mutation is predicted to cause a frameshift and a protein truncation eliminating the last two SH3 domains. Interestingly, a closely related homolog of SH3PXD2B, SH3PXD2A, has previously been shown to bind to members of ADAMs (a disintegrin and metallopeptidase) and to be required for podosome formation. Therefore, we have evaluated the ability of SH3PXD2B to form protein-protein interactions and to influence podosomes. Using GST pull-down, we found that SH3PXD2B is associated with ADAM15 through its last SH3 domain. In addition, we have shown that SH3PXD2B localizes to podosomes in cSrc-transfected fibroblasts. These results suggest that Sh3pxd2b might be involved in the function of podosomes. In the eye, we speculate that SH3PXD2B may regulate podosomes of the trabecular meshwork, influencing intraocular pressure and contributing to the glaucoma phenotype.

Cell Migration II

1005/B213

Pharmacological Analysis of Endogenous Receptors in Non-Adherent Cells Using a Label-free Live-cell Based Impedance Assay.

J. Proctor, H. Tang, T. Tutana, Q. Chen, R. McGuiness, D. Gallant; MDS Analytical Technologies, Sunnyvale, CA

Traditional non-adherent cell based assays, such as chemotaxis assays, are low throughput, time consuming and require fluorescent labeling of cells and/or reagents. The objective in this study was to demonstrate that label-free impedance technology could be used to reliably measure pharmacology of receptors endogenously expressed by a variety of non-adherent cells including primary monocytes. U937, THP-1 and primary blood monocytes (PBMC's) were obtained commercially and cultured according to the manufactures' recommendations or used the day of harvest. The cells were then challenged with reference agonists that stimulate endogenous purinurgic receptors (U937 and THP-1) or chemokine receptors (PBMC). Agonist responses were measured on the CellKey™ System using both the CellKey™ Standard 96W microplate and the Small Sample 96W microplate. The CellKey™ System uses an impedance-based technology to accurately measure endogenous receptor activation on non-adherent primary cells and cell lines. We show that this impedance-based platform reliably measured robust pharmacology for all cells tested. For monocyte chemotactic protein 1 (MCP-1), we determined an EC50 values ranging from 181 to 283 pg/mL at a %CV value of 9.2 to 9.4 using the Standard 96W microplate and the Small Sample 96W microplate respectively. The Small Sample plate required significantly fewer cells than the Standard microplate (40,000 compared to 225,000). This reduction in the number of cells required for the Small Sample plate was consistent across the non-adherent cells used. This Small Sample microplate allows this technology to be utilized more cost-effectively with primary cells in 96-well format.

1006/B214

Design and Fabrication of a Novel Migration Chamber to Study Cell Polarization, Force Generation and the 2D to 3D Migration Transition.

M. Breckenridge[1], H. Baskaran[2], T. Egelhoff[3]; [1]Physiology and Biophysics, Case Western Reserve University, Cleveland, OH, [2]Chemical Engineering, Case Western Reserve University, Cleveland, OH, [3]Cell Biology, Cleveland Clinic Foundation, Cleveland, OH

In order to study the isoform specific roles of nonmuscle myosin II in migration, novel migration chambers have been designed and fabricated using photo- and soft-lithography microfabrication techniques. Each chamber consists of two parts separated by a vertical barrier with multiple bays of gates with widths varying from 4µm to 25µm. The cells are plated on one part of the barrier while a chemoattractant is flowed through the other part of the barrier. These gates provide a gradient of resistance for cells to crawl across. The vertical wall allows for visualization of the cells as they migrate and are challenged by varying levels of resistance. Combining these chambers with manipulation of NM-II isoform expression levels will allow the study of NM-II isoform specific roles in cell polarization, force generation, and the 2D to 3D migration transition. The chemoattractant part of the chamber can be combined with a gradient generator to expose cells to stable chemoattractant gradients.

1007/B215

Mechanical Stimulation Enhances Cancer Cell Invasion into 3-Dimensional Matrices.

S. Menon, K. A. Beningo; Biological Sciences, Wayne State University, Detroit, MI

Metastatic cells migrate from the site of the primary tumor, through the stroma, into the blood and lymphatic vessels, finally colonizing various other tissues to form secondary tumors. Numerous studies have been done to identify the stimulus that drives the metastatic cascade. This has led to the identification of multiple biochemical signals that promote metastasis. However information on the role of mechanical factors in cancer metastasis is limited. Interestingly, the tumor microenvironment is rich in highly contractive cells responsible for the extensive remodeling and production of the dense extracellular matrix surrounding the cancerous tissue. We hypothesize that the mechanical forces produced by cells in the tumor microenvironment contribute to the invasion efficiency of metastatic cells. To test our hypothesis, we have designed a three-dimensional collagen matrix assay. Magnetic beads embedded within the matrix are used to induce the mechanical stimulus needed to test our hypothesis. Surprisingly, we have discovered a significant difference in the extent of invasion in mechanically stimulated verses non- stimulated culture environments. Furthermore, this mechano-invasion is dependent upon substrate protein composition, and influenced by compliance and matrix thickness. We conclude that mechanical stimulation is sufficient to enhance the invasive capacity of cancer cells in-vitro. We propose that non-cancerous cells located within the tumor micro-environment are capable of providing the necessary mechanical stimulus by tugging on the extracellular-matrix surrounding the tumor and are currently quantifying this possibility. This knowledge could provide a novel prognostic indicator or potential therapeutic target for cancer treatment.

1008/B216

Understanding and Directing Cell Motility on 2-D Micron-scale Patterns.

K. Kushiro, S. Chang, A. Asthagiri; California Institute of Technology, Pasadena, CA

Over the past decade, cell motility on 2-D surfaces has been extensively studied to unravel various cell-ECM interactions that affect cell migration. More recently, use of micron- and nano-scale patterns have emerged to study the effect of manipulating focal adhesion formation on cell morphology and adhesion. However, very limited work has been undertaken to study and control cell motility using these patterns. Here we present micron-scale, teardrop-based patterns to direct cell motility on 2-D surfaces. We observe that MCF-10A mammary epithelial cells exhibit an unnaturally high tendency to jump from the thin end of a teardrop pattern in a certain configuration, in addition to enhanced persistence and migration speed; this tendency and motility enhancement was lost when the surface pattern was slightly altered. We identify and test key pattern parameters, such as gap distance, positioning and shape, which can be used to precisely fine-tune the direction and motility of cells on these teardrop-based patterns. We further study the lamellipodia extensions of cells on the patterns and explain the observed directional biases and motility enhancements due to the subtle differences in the aforementioned parameters. The result of this study

will be useful in tissue engineering applications to direct cell movement on lab-on-a-chip devices using patterns and also to understand behavior of cells in various 2- and 3-D environments with different geometric constraints.

1009/B217

Cancer Cell Migration: Substrate Mechanics and Intracellular Mechanisms.

E. Mylona; Institute of Applied and Computational Mathematics, Foundation for Research and Technology-HELLAS, Heraklion, Greece

Cancer cell metastasis is guided by many chemical cues. Whether the mechanical properties of the immediate microenvironment can also direct cancer cell migration is largely unknown. We hypothesized that tumor cell migration will be directed by substrate rigidity. Cells from a human fibrosarcoma cell line (HT1080) were placed on collagen I-coated polyacrylamide gels. Gel composition was manipulated to approximate the rigidity of physiological (7 kPa) or malignant connective tissue (20 kPa). Collagen-coated polystyrene (PS) was used as control substrate. HT1080 cell migration was determined via phagokinetic tracks formed on a thin layer of 200 nm fluorescent latex beads for 24h. Compared to the compliant 7 kPa substrate, tumor cell migration distance and speed were 1.8- and 2.9-fold increased on the 7 and 20 kPa substrates, respectively. To determine cancer cell mechanotaxis, that is migration toward rigid surfaces, we assessed the phagokinetic tracks of cancer cells on a substrate rigidity gradient established between the 7 and 20 kPa gel polymers. HT1080 cells migrated directionally toward the stiffer end of the gradient, whereas cells from the stiffer end moved at the edge of the gradient without crossing toward the compliant substrate. Phalloidin staining revealed that actin fibers were well formed and organized on 20 kPa and PS substrates, albeit absent on the 8 kPa substrate. Kymographs of cell membranes showed that the 7 kPa substrate supported the formation of short lamellipodia, whereas long and persistent lamellipodia were present on the stiffer substrates. Immunocytochemistry for CD44 showed that the protein was prominently localized at the edge of lamellipodia on the stiffer substrates and omnipotent but clustered on the membranes of cells exposed to the compliant substrate. Together, these data signify that substrate rigidity biases tumor cell migration and suggest a potential involvement of CD44 in the process.

1010/B218

Substratum-dependent Effects of ROCK and Myosin Inhibitors during 2D and 3D Migration of Breast Epithelial Cells.

S. R. Gehler[1], K. I. Hulkower[2], R. L. Herber[2], P. Keely[1]; [1]Pharmacology, University of Wisconsin, Madison, WI, [2]Platypus Technologies, LLC, Madison, WI

Tumor metastasis is a multi-step process that requires cells to migrate through interstitial tissue to other areas of the body. Both Rho kinase (ROCK) and myosin are important regulators of cell migration and invasion, but whether ROCK and myosin have similar effects on cell migration on substrata comprised of different extracellular matrix (ECM) proteins or in the context of 2D and 3D environments requires further investigation. We propose that cell migration in 2D versus 3D environments is differentially regulated by ROCK and myosin in a substratum-dependent manner. Breast epithelial cells were plated into the OrisTM migration assay, a 96-well plate in which each well contained a silicone stopper that occluded cell attachment in the central part of the well. Once cells reached confluency, the stopper was removed and migration into the central region of the well was assessed. First, MDA-MB-231 and NMuMG breast epithelial cells cultured on collagen undergo more 2D migration and more robust focal adhesion formation compared to those cells plated on fibronectin. Second, pharmacological inhibition of ROCK or myosin II reduced MDA-MB-231 cell migration on both collagen and fibronectin which also correlated to a reduction in focal adhesion formation. Interestingly, NMuMG cell migration in 2D was enhanced in the presence of either ROCK or myosin II inhibitors. Next, we investigated whether ROCK or myosin II inhibition had similar effects on 3D migration, by overlaying a collagen gel on the adherent cells following stopper removal. Consistent with 2D collagen migration, inhibition of either ROCK or myosin II reduced 3D collagen migration. However, contrary to 2D migration, inhibition of ROCK or myosin II reduced NMuMG migration into a 3D collagen gel. These results suggest that the properties of the ECM, including protein composition and matrix dimensionality, differentially regulate breast epithelial cell migration in response to inhibition of ROCK and myosin II.

1011/B219

EGF Receptor-Mediated Regulation of Directional Persistence in 3D Tumor Cell Migration.

H. Kim[1,2], T. W. Guo[1], A. P. Wu[1], F. Gertler[2], D. Lauffenberger[1,2]; [1]Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, [2]Koch Institute for Integrative Cancer Research, Massachusetts Institute of Technology, Cambridge, MA

Cell migration mediated by the epidermal growth factor (EGF) receptor and its ligands plays a vital role in invasion of many tumor types. Activation of the EGF receptor increase invasiveness in vivo mediated by its downstream effectors, but it remains unclear how resultant effects on the intrinsic cell motility machinery versus effects on extrinsic matrix properties integrate to regulate locomotion speed and/or directional persistence in a 3D environment. Elucidating this convolution is important for therapeutic targeting of tumor invasion, as regulatory pathways for intrinsic and extrinsic effects may not be coincident. Consequently, we performed a quantitative, single-cell imaging study of glioblastoma cell movement in 3D matrices and 2D substrata across a range of collagen densities. EGF induced an increase in cell speed and a strong, matrix density-dependent increase in directional persistence in 3D, in contrast to an increase in cell speed but a significant decrease in directional persistence in 2D. The discrepant result was not due to differential activity of Rac1, a previously revealed cell-intrinsic switch for persistence. While cell migration in 2D was independent of protease activity, lack of protease activity in 3D resulted in characteristically matrix-confined cell movements causing low persistent migration behaviors. Systematic variation of protease-mediated matrix degradation showed a strong correlation between matrix degradation and 3D directional persistence. Finally, over-expression of Mena, a cytoskeletal effector of EGF receptor signaling, leads to a drastic decrease in directional persistence mediated by random protrusion activity in 2D. But this characteristic behavior is masked in 3D matrices resulting in an increase in cell speed with no change in directional persistence. Therefore, EGF receptor-mediated 3D tumor cell migration results only partially from enhancement in cell intrinsic motility, with override of cell-intrinsic reduction of persistence by cell-extrinsic, protease- and matrix-mediated regulation of matrix steric hindrance.

1012/B220

Adhesion and Endocytosis in Neuronal Migration.

J. C. Shieh[1,2], B. T. Schaar[3], S. K. McConnell[2]; [1]Neurosciences Program, Stanford University, Stanford, CA, [2]Biology, Stanford University, Stanford, CA, [3]Neurosurgery, Stanford University, Stanford, CA

Neuronal migration is critical for the proper development of the nervous system, as demonstrated by the variety of mental disorders in humans caused by disruptions in migration. Morphologically distinct steps characterize the movements of migrating neurons: a single leading process extends ahead of the stationary cell soma, followed by the formation of a cytoplasmic dilation ahead of the nucleus, subsequent movement of the nucleus into the dilation and retraction of the cell rear. We hypothesize that the temporal and spatial regulation of adhesion is important for the proper progression of these steps. Because endocytosis has been implicated in the process of adhesion disassembly, we examined the potential role of endocytosis in the regulation of adhesion during neuronal migration. In neurons migrating in a three-dimensional matrix, adhesions are present at the tip of an extended leading process and in the dilation ahead of the nucleus. Components of the clathrin endocytic machinery are also localized to the dilation region of a migrating neuron, where they would be properly positioned to regulate adhesions in that region. We have tested the role of endocytosis in neuronal migration by individually disrupting multiple components of the endocytic pathway using both molecular genetic and pharmacological approaches both in vitro and in vivo. Endocytosis is critical for normal neuronal migration and may contribute to adhesion regulation.

1013/B221

Invasion of Transformed Epithelial Cells in a Three-dimensional Matrix.

W. Shih, S. Yamada; University of California, Davis, CA

Cell adhesion plays a crucial role in embryonic development, tissue regeneration, and tumor metastasis. In order to navigate through a dense extracellular matrix and between neighboring cells, these cellular rearrangements critically depend on the balance of forces along cell-extracellular and cell-cell contacts. Yet, little is known about molecular mechanisms by which cellular traction forces are generated and transmitted through adhesion proteins to the extracellular matrix. This is partly due to the lack of experimental analysis done to quantitatively measure cellular forces in a more physiologically relevant three-dimensional environment. To address this, we embedded fluorescent tracer particles in a three-dimensional collagen matrix to quantitatively analyze the deformation field of the matrix and traction forces of invasive cancer cells. Unlike other cell types, the transformed epithelial cells displayed unique cell polarization: the nucleus was positioned at the leading edge of the membrane extension and the centrosome located at the trailing edge. As indicated by the movement of tracer particles, this leading edge exerted significant traction force onto the matrix. In fact, the deformation of the collagen network was the highest at the leading and trailing edge of the cell. The addition of Y27632, a Rho kinase inhibitor, caused the relaxation of the collagen network, which suggests that the traction force was generated by actin-myosin contraction. Our novel approach to quantitatively analyze cell invasion in a three-dimensional matrix revealed a new mode of cell invasion that better mimics cell migration in vivo. Understanding how cancer cells generate and transmit traction forces to the extracellular matrix or to neighboring cells will guide the search for therapeutic drugs that can prevent the invasion of cancer cells, and ultimately inhibit metastasis.

1014/B222

Strain Energy of Invasive and Non-Invasive Carcinoma Cells in Three-Dimensional Collagen Gels.

T. M. Koch[1], S. Münster[1], C. T. Mierke[1], J. P. Butler[2], B. Fabry[1]; [1]Department of Physics, University of Erlangen-Nuremberg, Erlangen, Germany, [2]Physiology Program, Harvard School of Public Health, Boston, MA

Cell invasion through a dense 3-dimensional matrix is believed to sensitively depend on the ability of cells to generate traction forces. To quantify the role of cell tractions during invasion, we measured the strain energy of invasive (MDA-MB-231 breast carcinoma, A-125 lung carcinoma) and non-invasive (MCF-7 breast carcinoma, A-431 lung carcinoma) cells cultured in a 3-D reconstituted collagen gel (shear modulus G'=100 Pa, 500 µm thickness, average mesh size 1.6 µm). Invasive cells assumed an elongated spindle-like morphology, non-invasive cells a rounder shape. In both cases, cells locally contracted the gel. The undeformed state of the gel was obtained after addition of the actin-disrupting drug cytochalasin-D. Gel deformations were measured by tracking the 3-D spatial positions of fluorescent beads (1 µm diameter) embedded in the gels. The bead positions served as nodes for a finite element tessellation. From the local strain of each element and the elasticity of the collagen, we computed the local strain energy stored in the collagen gel surrounding the cell. This technique was verified by indenting the surface of the gel with a steel sphere (100 µm diameter, gravitational force 35.4 nN). The strain energy of invasive lung carcinoma cells was (17±6) pJ (n=25) and significantly higher than the strain energy of non-invasive lung carcinoma cells of (9±2) pJ (n=25). This finding was more pronounced for breast carcinoma cells with strain energies of (13±4) pJ (n=35) for invasive vs. (2±1) pJ (n=45) for non-invasive cells. These results demonstrate that traction generation of carcinoma cells in 3-D plays an important role during invasion.

1015/B223

Linking Cell Shape Changes and Traction Forces in Migrating Amoeboid Cells.

B. Alonso-Latorre[1], R. Meili[2], E. E. Bastounis[1,2], J. C. del Alamo[1], R. Firtel[2], J. C. Lasheras[1]; [1]Mechanical & Aerospace Engineering, University of California San Diego, La Jolla, CA, [2]Cell & Developmental Biology, University of California San Diego, La Jolla, CA

Amoeboid motility is a complicated process requiring the regulated activity and localization of many molecules, which results in the cyclic repetition of a relatively small repertoire of shape changes (motility cycle). The aim of this study was to identify the dominant shape changes in this repertoire, and more importantly, the biochemical and mechanical processes associated with them. For this purpose, we acquired time-lapse recordings of cell shape and traction forces of Dictyostelium cells migrating on deformable substrates. We applied Phase Average Analysis (PAA) by splitting each motility cycle into four stages (protrusion, contraction, retraction and relaxation) according to the oscillations in cell length. We used Principal Component Analysis (PCA) to identify the most significant cell shape changes occurring during motility. A novel mathematical representation of cell shape allowed us to obtain maps of the traction forces associated with each shape change. Using wild-type cells (wt) as reference, we investigated the effect of altering myosin II activity by studying myosin II null cells (mhcA-) and essential light chain null cells (mlcE-). Results from PAA revealed that wt, mlcE- and mhcA- cells implemented a similar motility cycle. However, the duration of all four phases of the cycle was higher in mlcE- and mhcA- than in wt cells. The spatial distribution

of traction forces of wt and mlcE- cells were similar and more polarized than in mhcA- cells. However, there were no significant differences in the magnitude of the forces between wt and mhcA- cells. PCA showed that only three cell shape modes are necessary to represent 67% of the variance in area of mlcE- and mhcA- and wt cells. These shape modes are dilation/elongation, a half-moon shape and a bulging of the front or back. The second of these modes represents sideways protrusion and retraction, is associated to a lateral asymmetry in cell traction forces, and is significantly less important in mhcA-. Taken together, these results indicate that the mechanical cycle of traction stresses and cell shape remains similar but is slowed down when myosin function is lost, probably due to a reduced control on the spatial organization of the traction stresses.

1016/B224

An In-Vitro Correlation of Mechanical Force and the Degree of Metastatic Potential.

I. Indra[1], U. Thirumurthi[1], V. Undyala[1], M. Dembo[2], K. A. Beningo[1]; [1]Biological Sciences, Wayne State University, Detroit, MI, [2]Biomedical Engineering, Boston University, Boston, MA

Mechanical forces have a major influence on cell migration and are predicted to significantly impact cancer metastasis, yet this idea is currently ill defined. In this study we have asked if changes in mechanical forces and migratory properties occur as tumor cells progress in the metastatic spectrum towards aggressive metastasis. For this purpose, five murine breast cancer cell lines derived from the same primary tumor, but possessing variable metastatic potential, were tested for adhesion strength, traction force, migration rate, and focal adhesion organization. Using Traction Force Microscopy we were surprised to find an inverse relationship between traction force and metastatic potential, such that force production decreased as the metastatic potential increased, with the exception of the most aggressive cell line in which traction forces returned to wild-type levels. Consistent with this observation, adhesion strength, as determined by centrifugation assays, exhibited an identical profile to the traction data. Surprisingly however, migration rates and persistence were relatively low in the entire panel with the exception of the most aggressive cell line, which moved very efficiently. A count of adhesions per cell type indicated a general reduction in the number of adhesions throughout the panel, but no difference in the maturation as determined by the ratio of nascent to mature adhesions. We conclude that traction and adhesion forces do indeed change as tumor cells progress in metastatic capacity, and surprisingly this correlation does not extend to changes in focal adhesion organization or migration rates.

1017/B225

MIM Controls Cell Migration through Spatiotemporal Regulation of Guidance Receptor Signaling.

G. A. Quinones[1,2], J. Jin[1,2], A. Oro[1,2]; [1]Cancer Biology Program, Stanford University, Stanford, CA, [2]Program in Epithelial Biology, Stanford University, Stanford, CA

Directed cellular migration is a critical aspect of normal development that facilitates the coordinated movement of individual cells, cell clusters and neural networks during embryogenesis. Cell migration is also a key event during wound healing in the adult and migratory abnormalities have been implicated in mental retardation, birth defects and cancer metastasis. We and others have previously shown that vertebrate Missing in Metastasis (MIM) is a defining member of the I-BAR subfamily of lipid binding, cytoskeletal regulators whose levels are altered in metastatic cancers. Here we provide the first in vivo genetic evidence that I-BAR proteins regulate directed cell migration. Drosophila MIM (dMIM) is required for guided border cell migration in the ovary, with loss of dMIM resulting in the failure to polarize the leading edge. We used live-cell imaging of moesin-GFP expressing migrating cells in the intact ovary to analyze membrane dynamics in a native environment. Our studies demonstrate that dMIM does not affect general actin cytoskeletal dynamics, but rather the ability of the cell to send directional cell extensions toward the guidance cue. MIM also alters the temporal and spatial growth factor signaling through inhibition of receptor-mediated endocytosis. This is accomplished in part by assembling a complex of proteins that balances the endocytosis-promoting activities of the cytoskeletal protein cortactin. These studies suggest that the I-BAR family constitutes a novel steering mechanism used to interpret local migratory cues.

1018/B226

Srgap2 Regulates Neuronal Migration through the Ability of Its F-BAR Domain to Induce Filopodial-Like Membrane Protrusion.

S. Guerrier[1,3], J. Coutinho-Budd[1,3], T. Sassa[1,3], A. Frost[2], V. Unger[2], F. Polleux[1,3]; [1]Pharmacology, UNC-CH, Chapel HIll, NC, [2]Biochemistry and Biophysics, Yale School of Medicine, New Haven, CT, [3]Neuroscience Research Center, UNC-CH, Chapel Hill, NC

During brain development, proper neuronal migration is critical for the establishment of functional neuronal circuits. Here we report that srGAP2 is a novel, negative regulator of neuronal migration in the cerebral cortex through the ability of its F-BAR domain to regulate the formation of filopodial-like membrane protrusions. Unlike previously characterized domains of this family, the F-BAR domain of srGAP2 induces filopodial-like membrane protrusions resembling those induced by I-BAR domain-containing proteins. In vitro, induction of filopodia has been shown to decrease the rate of migration and leading edge persistance in non-neuronal cell lines. Similarly, reduction of srGAP2 levels using shRNA-mediated knockdown reduces the complexity of their leading process and increases the rate of neuronal migration in vivo. Conversely, overexpression of srGAP2 or its F-BAR domain significantly inhibits the migration of neurons by increasing the dynamic and decreasing the stability of their processes. Finally, expression of a truncated F-BAR domain that can localize to the membrane but cannot elicit filopodial-like membrane protrusions does not inhibit neuronal migration. These results highlight the importance of regulating filopodial-like membrane protrusions at the leading process of neurons for their proper migration.

1019/B227

IGF-I Secreted by Osteoblasts Acts as a Potent Chemotactic Factor for Osteoblasts.

M. Nakasaki[1], K. Yoshioka[1], Y. Miyamoto[2], T. Sasaki[3], K. Itoh[1]; [1]Biology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan, [2]Immunology, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan, [3]Pathology and Immunology, Akita University School of Medicine, Akita, Japan

Osteoblast recruitment to the site of future bone formation is essential for skeletal development, bone remodeling and fracture healing. A number of factors associated with bone tissue have been reported to induce directional migration of osteoblasts but the mechanism remains to

be clarified. In this study, to explore a major chemotactic factor(s) for osteoblasts, we examined the serum-free medium conditioned by MC3T3-E1 osteoblast-like cells for its ability to induce osteoblast migration. Employing sequential chromatography and tandem mass spectrometry analysis, we purified and identified IGF-I as a potent chemotactic factor from the conditioned medium. IGF-I induced cell migration of both MC3T3-E1 cells and primary mouse osteoblasts, and checkerboard analysis revealed that IGF-I markedly induced directional migration (chemotaxis) of osteoblasts. Neutralization of mouse IGF-I with monoclonal antibodies resulted in delayed osteoblast monolayer wound healing and cellular polarization but addition of human IGF-I reversed these effects. IGF-I also promoted cell spreading on fibronectin in an integrin β1-dependent manner. IGF-I induced Akt and Rac activation and localized accumulation of phosphatidylinositol 3,4,5-triphosphate (PtdIns (3,4,5)P3) at the membrane in osteoblasts. The phosphatidyl inositol 3 kinase (PI3K) inhibitor LY294002 inhibited IGF-I-induced cell migration and wound healing. Together, the results suggest that IGF-I secreted from osteoblasts in the bone tissue is a potent chemotactic factor that may play a major role in recruitment of osteoblasts during bone formation.

1020/B228

Role of MYADM in Cell Morphology and Migration.

J. Aranda[1], N. Reglero[1], B. Marcos[1], A. Ridley[2], J. Millan[1], M. A. Alonso[1]; [1]Cell Biology and Inmunology, Centro de Biologia Molecular Severo Ochoa, Madrid, Spain, [2]Randall Division of Cell and Molecular Biophysics, King`s College London, London, United Kingdom

Cell polarity is characterized by the establishment of morphological, molecular and functional asymmetry required in essential processes, from epithelial formation or neural differentiation to cell migration. There are two main mechanisms that contribute to establish polarity at the plasma membrane. First, cell membranes have the ability to form domains of specific lipid composition that selectively cluster proteins in order to regulate signalling processes. These domains are heterogeneous and are commonly known as membrane or lipid rafts. Second, the underlying cytoskeleton contributes also to protein segregation by interacting directly with transmembrane proteins and/or modifying the distribution of membrane rafts. Membrane rafts and the actin cytoskeleton act co-ordinately during processes that require polarization, namely cell motility, but the underlying mechanism regulating this coordination is poorly understood. This is partly because the protein machinery that regulates the function of membrane rafts is almost unknown. Amongst proteins candidates to constitute the scaffold of membrane rafts are proteins from the MAL family, which contain MARVEL domains, involved in membrane apposition. We present in this meeting data about the functional characterization of the myeloid-associated differentiation marker protein (MYADM), a new member of the MAL family of unknown function. We show that MYADM contains two MARVEL domains, is an integral component of membrane rafts and is ubiquitously expressed. MYADM localizes to actin-rich structures in cell-to-cell junctions in confluent epithelial monolayers and to lamellipodium-like structures in migrating cells. Functional analysis using a siRNA-based approach indicates that MYADM regulates cell morphology, polarization and migration in various cell types. Our data also suggest that MYADM is a structural protein involved in the interaction between the plasma membrane and the actin cytoskeleton, and thereby regulate cell polarity and migration.

1021/B229

ErbB-2 Signaling Promotes Matriptase Zymogen Activation in Human Prostate Cancer Cells.

M. Lee[1], T. Cheng[1], C. Deng[1], S. Wu[1], C. Lin[1], C. Huang[1], J. Liao[1], C. Ko[1], C. Lin[2]; [1]Biochemistry and Molecular Biology, College of Medicine, National Taiwan University, Taipei, Taiwan, [2]Biochemistry and Molecular Biology, University of Maryland School of Medicine, Baltimore, MD

Deregulation of ErbB-2 signaling and matriptase activity have been shown in human cancer lesions. In this communication, we further investigated the involvement of ErbB-2 signaling in matriptase zymogen activation of human prostate cancer cells and the role of matriptase in ErbB-2-induced prostate cancer malignancy. Highly tumorgenic human androgen-independent prostate cancer PC-3, DU145, and LNCaP C-81 cells, exhibit an aggressive phenotype with a hypertyrosine phosphorylated ErbB-2 and concurrently had increased in matriptase activity, compared to lowly-tumorigenic human androgen-sensitive prostate cancer LNCaP C-33 cells. Ectopic expression of ErbB-2 in LNCaP C-33 cells resulted in an increased activation of matriptase. The inhibition of ErbB-2 activity by a specific inhibitor AG825 or an ErbB-2 shRNA knockdown led to a decrease of matriptase activity in PC3 or LNCaP C-81 cells which had an activated matriptase. Moreover, in further investigation of downstream pathways, our data showed that a PI3K inhibitor LY294002 but not an MEK inhibitor PD98059 suppressed the level of ErbB-2 induced matriptase activity in ErbB-2-overexpressing LNCaP C-33 cells. Conversely, reduction of matriptase protein level had no significant effect on ErbB-2-promoted cell proliferation, motility, and transmigration of human prostate cancer cells. Interestingly, reduction of matriptase activation in ErbB-2-overexpressing LNCaP C-33 cells led to a decrease of cell invasion, suggested that matriptase play an important role in the ErbB-2 signal pathway to promote prostate cancer cell invasion. Taken together, our data indicate that the ErbB-2 signal pathway via PI3 kinase signaling is involved in up-regulating matriptase zymogen activity which contributes to prostate cancer cell invasion.

1022/B230

Mupp1 and Patj, Two Closely Related Proteins, Interact with the Crb3A Complex and Play Different Roles in Polarized Cell Migration.

E. Assémat, E. Crost, D. Massey-Harroche, A. Le Bivic; UMR 6216 IBDML, CNRS, Marseille, France

Recent studies have highlighted that polarity complexes are not only important for the organization of epithelium into a tissue but also for regulation of cellular processes such as differentiation, motility and growth. The Crb3A complex composed of Crb3A, Pals1 and Patj proteins is involved in epithelial polarity establishment and maintenance of tight junction. Mupp1 is structurally very similar to Patj; both proteins have multiple protein-protein interaction domains: a L27 domain at their N-terminus followed by several PDZ domains. We showed by immunoprecipitation experiments that Mupp1 interacts with Pals1, a protein of the conserved Crb3A/Pals1/Patj apical complex suggesting the existence of another complex composed of Crb3A/Pals1/Mupp1 in epithelial cells. The role of this complex is not clear and by using RNA interference we showed that Mupp1, Pals1 and Patj are co-regulated and co-stabilized in the cell. Down-regulation of Pals1 induces both a loss of Patj and Mupp1. The mechanisms underlying this regulation are under investigation. Loss of Mupp1 affects Golgi reorientation but, as opposed to Patj, not migration in mammary epithelial cells by using a wound-induced migration assay. Together our data indicate that

Patj and Mupp1 belong to two structurally independent but functionally linked complexes with different downstream effectors in cell migration events.

1023/B231

Indispensable Role of Phospholipase C Gamma 2 in Regulating Cell Migration and Cell-Cell Interactions in Dendritic Cells via the Rac1 Pathway.

V. Cremasco[1], D. B. Graham[2], M. Cella[2], M. Kisseleva[3], R. Faccio[1]; [1]Department of Orthopedics, Washington University School of Medicine, St. Louis, MO, [2]Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO, [3]Department of Internal Medicine, Washington University School of Medicine, St Louis, MO

Dendritic cells (DCs) are highly plastic cells, which capture antigen in peripheral tissues and migrate at great speeds to lymph nodes, where they dynamically interact with and activate T cells. Given the importance of the PLCγ pathway in the induction of inflammatory responses associated with arthritic diseases, we hypothesized that PLCγ2 might modulate the function of DCs by regulating cytoskeletal dynamics. Confirming our hypothesis, we observed that antigen pulsed PLCγ2-/- DCs were unable to promote T cell activation in vivo. When we employed a competitive homing assay in vivo to assess cell recruitment to the lymph nodes, we observed that the number of PLCγ2-/- DCs reaching the lymph nodes was dramatically decreased compared to WT cells (PLCγ2-/-=0.03±0.01% and WT=0.23±0.07% of total lymph node cells). Consistent with this finding was our in vitro observation that PLCγ2-/- DCs were unable to migrate towards the chemokine CCL21, which is known to recruit DCs to the lymph nodes (PLCγ2-/-=16±4 and WT=60±17 cells/field). In addition, we investigated the importance of PLCγ2 to modulate the cytoskeletal changes accompanying the interaction of DCs with T cells. Using time-lapse video microscopy, we observed that PLCγ2-/- DCs appeared poorly motile and presented only few, small membrane extensions around their bodies compared to WT cells that instead formed numerous membrane protrusions to surround the adjacent T cells. Mechanistically, we found that PLCγ2 was required for CCL21-induced activation of Rac1, a small GTPase involved in membrane ruffle formation and cell migration, but not for Rho activation. Importantly, CCL21-dependent PKC phosporylation was dampened in PLCγ2-/- DCs, suggesting that the catalytic activity of the enzyme is required for Rac1 activation and actin-dependent events in DCs. Thus, for the first time, we demonstrate an essential role for PLCγ2 in DC function by modulating the cytoskeleton dynamics associated with cell migration and cell-cell interactions, during the course of an inflammatory response.

1024/B232

The Role of Icmt-Catalyzed Methylation in Rho Signaling and Migration.

I. Cushman, P. J. Casey; Pharmacology and Cancer Biology, Duke University, Durham, NC

A number of proteins that play key roles in biological regulatory events undergo a complex series of post-translational modifications that are directed by the presence of a so called CaaX motif at their carboxyl-terminus. The CaaX motif directs proteins through three enzymatic steps resulting in a prenylated and methylated carboxyl-terminal cysteine. This protein modification pathway plays a significant role in cancer biology because many oncogenic proteins contain CaaX motifs and require modification by this pathway. The final step in CaaX protein processing is methylation of the carboxyl-terminus by isoprenylcysteine methyltransferase (Icmt). Cysmethynil, a specific Icmt inhibitor discovered in our lab, is able to inhibit Ras-mediated signaling, cell growth, and oncogenesis. Additional studies by our lab and others have shown that an impact on Rho function may be at least partly responsible for the anti-tumor effects of Icmt inhibition. Due to the prevalence of Rho GTPases in biological processes, including carcinogenesis, and the fact that CaaX processing is a proven target for therapeutic drug design; we are utilizing cysmethynil to determine the biological consequences of Rho methylation. Our results indicate that inhibition of methylation reduces cell proliferation and migration of the highly metastatic MDA-MB-231 breast cancer cell line, which expresses high levels of RhoA. In addition, cell adhesion and cell spreading are also significantly inhibited by cysmethynil. To examine the mechanism of cysmethynil-mediated inhibition of migration we focused on RhoA and Rac1, which are important mediators of cell migration. Inhibition of Icmt with cysmethynil significantly decreases the activation of both RhoA and Rac1. Loss of activation may be due to increased RhoGDI binding in the absence of methylation. Furthermore, in the absence of Icmt activity the addition of exogenous RhoA or Rac1 is able to partially rescue directed and random migration, respectively. We have shown a role for Icmt-mediated methylation in cell migration and advanced our understanding of the biological consequences of Rho methylation.

1025/B233

p120 Catenin is Required for Gastrulation in Zebrafish Embryos.

M. B. Hille, R. Walsh, C. Muerdter, C. Hsu, P. Sanchez, T. Chen, M. Nguyen, N. Bahrami, J. Mytton; Biology, University of Washington, Seattle, WA

We are investigating the roles of cadherins and p120 catenin during the gastrulation of zebrafish embryos. Presomitic mesodermal cells of gastrulating zebrafish embryos contact each other through transmembrane cadherins (Crawford et al 2003). p120 catenin is known to bind to the juxtamembrane position of cadherins to stabilize adhesion and to activate Rac GTPase in the cytosol, which stimulates motility (Erez et al 2005; Xiao et al, 2007). In order to determine the role of cadherins during gastrulation, we knocked down p120 catenin delta1 with an antisense morpholino oligonucleotide (MO) designed to a splice site of the gene. Splice MOs block the synthesis of new mRNAs, but allow translation of stored maternal mRNAs. Knocking down p120 catenin slowed or delayed convergence of the dorsal mesoderm: elongated somites were observed at the 9-somite stage in 90% of embryos injected with 4-8 ng splice MO. At later stages, p120 catenin knockdown caused shortened tails and reduced yolk extensions. To demonstrate that knocking down p120 catenin is responsible for these defects, we coinjected the splice MO with a p120 catenin delta2 mRNA homolog from *Xenopus*. The concentration of *Xenopus* p120 catenin mRNA used for injection caused no observable defects in zebrafish gastrulation when injected alone, but rescued all of the observed defects. We used RT-PCR to determine the amounts of the p120 catenin isoforms, p120 catenin delta1, p120 catenin delta2, and ARVCF, which is known to rescue p120 catenin knockdown in *Xenopus* embryos (Fang et al 2004). p120 catenin delta1 mRNA is expressed in high quantities in 500-cell embryos and declines gradually through the 12-somite stage. ARVCF mRNA occurs at about half the concentration of p120 catenin delta1 at 500-cells but declines abruptly by epiboly, while p120 catenin delta2 is negligibly present at all stages. Taken together, these results suggest

that p120 catenin regulation of cell migration is essential to convergence and extension: the extended somites and shortened tail observed in p120 catenin knockdown embryos suggested a failure or slowing of mesodermal migration. Likely both the turnover of cadherin adhesion and the activation of polarized Rac GTPase are required.

1026/B234

Interplay between Aquaporin-9, α-Dystrobrevin and β2-Integrins in Attaching Neutrophil-like HL60 Cells.

T. Karlsson[1], V. V. Borutinskaite[1,2], R. Navakauskiene[2], K. Magnusson[1], V. Loitto[1]; [1]Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden, [2]Department of Developmental Biology, Institute of Biochemistry, Vilnius, Lithuania

Aquaporins (AQPs) have proved important in cell adhesion and cell motility, but the effects of AQPs on cell-substratum interactions are distinct depending on cell type, substratum and type of AQP. Because AQP4 has been shown to co-localize with the dystrophin-associated protein (DAP) complex known to be involved in cell adhesion, we wanted to address this issue further. To assess this we studied how over-expression of AQP9 affected the adhesiveness in neutrophil-like HL60 cells. AQP9 is an aquaglyceroporin, where the phosphorylation on serine11 relates to the induction of filopodia in fibroblasts, which increases the surface area and potential substratum interactions. Using various imaging techniques and neutrophil-like HL60 cells, we have tested how over-expression of AQP9 affects cell adhesion, β_2-integrin presentation and whether AQP9 co-localizes with α-dystrobrevin, which is a known member of the DAP-complex. We found that AQP9 expression enhanced cell-substratum adhesion, requiring accessibility of serine11 for phosphorylation. We also observed an increase in β_2-integrin expression and a very distinct co-localization between AQP9 and α-dystrobrevin. We suggest that the enhanced adhesive ability is promoted by the increase in β_2-integrin expression and surface area that follows induction of filopodia by AQP9, which is likely strengthened by the co-localization with the DAP-complex.

Cilia & Flagella II (1027 – 1044)

1027/B235

Intraflagellar Transport Particle Size Scales Inversely with Flagellar Length: Revisiting the Balance-Point Length Control Model.

B. Engel, W. Ludington, W. Marshall; University of California San Francisco, San Francisco, CA

The assembly and maintenance of eukaryotic flagella is regulated by intraflagellar transport (IFT), the process of bidirectional protein traffic within the flagellum. IFT particles, linear arrays of IFT proteins and axonemal precursors, are transported by kinesin-II to the site of flagellar assembly at the tip, and returned to the base by cytoplasmic dynein-1b. Current models have failed to reconcile the observed constant speed and frequency of particle transport with the flagellar assembly rate, which decreases as a function of flagellar length. Using total internal reflection fluorescence (TIRF) microscopy tuned to allow imaging completely through the flagella of Chlamydomonas reinhardtii, we quantified protein traffic during flagellar regeneration and determined that anterograde IFT particles in short flagella are composed of more KAP (kinesin associated protein) and IFT27 proteins than particles in long flagella. This length-dependent remodeling of particle size is consistent with the kinetics of flagellar regeneration and supports a revised balance-point model of flagellar length control, where IFT particle size, rather than speed or frequency, modulates the rate of flagellar assembly.

1028/B236

Chlamydomonas IFT20 is required for the Assembly of IFT Particles and Flagella.

B. Craige[1], D. Cochran[1], M. J. Chapman[3], J. Follit[2], Y. Hou[1], K. Lechtreck[1], S. Takada[4], G. Pazour[2], G. Witman[1]; [1]Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA, [2]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, [3]Department of Biology, College of the Holy Cross, Worcester, MA, [4]Department of Biochemistry and Molecular Biophysics, Washington University School of Medicine, St. Louis, MO

Intraflagellar transport (IFT) is the bidirectional movement of IFT particles along the length of flagella and cilia, and is required for the assembly and maintenance of these organelles. The protein IFT20 is part of IFT complex B, and in mammalian cells IFT20 localizes to basal bodies, cilia, and the Golgi apparatus. Here we describe a Chlamydomonas insertional mutant that contains a complete deletion of the IFT20 gene. The IFT20 null cells mostly lack flagella; however, rare cells display short or stumpy flagella that have defects in the transition zone. Constructs encoding HA- and GFP-tagged IFT20 rescue the mutant phenotype. The tagged proteins co-localize and co-immunoprecipitate with other IFT complex B proteins, but do not display Golgi localization. The localization pattern was confirmed in wild-type cells using antibodies to endogenous IFT20. In contrast to deletions in IFT88, IFT140, and IFT57, loss of IFT20 does not decrease the whole cell levels of IFT172, IFT139, IFT57, IFT46, or IFT27. However, loss of IFT20 alters the sedimentation pattern of IFT proteins in sucrose gradients. These data indicate that IFT20 is required for the assembly of functional IFT particles.

1029/B237

Function of IFT20 in Mouse Kidney and Retina.

B. Keady, J. Jonassen, J. Follit, J. SanAgustin, G. Pazour; Molecular Medicine, UMass Medical School, Worcester, MA

The IFT20 subunit of the IFT particle is localized to the Golgi complex where it is likely involved in the trafficking of ciliary membrane proteins (Follit et al., Mol.Biol.Cell 17:3781-92). To understand the function of IFT20, we generated a floxed allele in mouse and deleted IFT20 specifically in kidney collecting ducts and retinal cone cells. Deleting IFT20 in kidney collecting duct cells prevented cilia formation and promoted rapid postnatal cystic expansion of the kidney. Dividing collecting duct cells in early stages of cyst formation fail to properly orient their mitotic spindles along the tubule, while non-dividing cells improperly position their centrosomes. At later stages, cells lacking cilia have increased canonical Wnt signaling and increased rates of proliferation. Thus, IFT20, likely acting through cilia, plays a key role in coupling extracellular events to cell proliferation and differentiation. Deleting IFT20 in cone cells caused red-green opsin to accumulate in the inner segments and at the ribbon synapse followed by a progressive loss of the cone cells. Rods did not appear to be affected as rod

specific opsin (rhodopsin) was localized normally in these animals. These data indicate that IFT20 is required for the development and maintenance of cone outer segments and suggest that IFT20 plays a role in transport of RG opsin to their outer segments.

1030/B238

Mechanism of Suppression of the Chlamydomonas Intraflagellar Transport (IFT) Mutant ift46.

Y. Hou, G. Witman; Cell Biology, University of Massachusetts Medical School, Worcester, MA

IFT, the bi-directional movement of particles within flagella, is required for flagellar assembly. The IFT particles of *Chlamydomonas* are composed of ~16 proteins, organized into complexes A and B. We previously reported partial suppression of the insertional mutant *ift46*, which is null for complex B protein IFT46 and forms very short flagella lacking outer arms. Under stress conditions only, the suppressor, $Sup_{ift46}1$, forms longer flagella that still lack outer arms. RT-PCR showed that the 3' end of the IFT46 gene is transcribed in $Sup_{ift46}1$ but not in the null mutant. To understand the mechanism of suppression, we cloned the 5' end of this transcript and sequenced the corresponding genomic region of $Sup_{ift46}1$. We found that insertion of a MRC1 transposon at that site allows transcription of the downstream part of the IFT46 gene encoding amino acids 105-344. To see if the transcript is functional, we made a polyclonal antibody to the predicted truncated IFT46 protein. This antibody recognizes a band of the predicted size in whole cell lysates of $Sup_{ift46}1$ but not wild-type or null mutant cells. This truncated protein was also detected in $Sup_{ift46}1$ flagella formed under the stressed condition but not in wild-type flagella. Sucrose gradient analysis revealed that the truncated IFT46 is incorporated into complex B in $Sup_{ift46}1$ flagella. To see if the expression of this truncated IFT46 protein could cause the suppression, we created a construct that expresses a protein similar to the truncated IFT46 protein and transformed it into the null mutant. 27 out of 288 transformants had the same phenotype as $Sup_{ift46}1$. Therefore, expression of a truncated IFT46 is required for the partial suppression. To understand the requirement for stress conditions, we compared $Sup_{ift46}1$ cells under stress and non-stress conditions. Stressed $Sup_{ift46}1$ cells have more truncated IFT46 protein and other IFT complex B proteins than non-stressed $Sup_{ift46}1$ cells. We hypothesize that stress induces expression of a protein that stabilizes complex B containing truncated IFT46. We are comparing the flagella membrane + matrix fractions from wild-type and $Sup_{ift46}1$ cells grown under the stressed condition and have found at least one difference.

1031/B239

Interaction Analysis of IFT Complex A Subunits IFT43 and IFT121.

R. H. Behal, A. Jones, M. S. Miller, C. Rea, D. Cole; Micro, Molec Biol & Biochemistry, University of Idaho, Moscow, ID

Intraflagellar transport (IFT) is a strongly conserved process required for the assembly and function of most eukaryotic cilia and flagella. Excluding the IFT motors, IFT particles consist of multiple copies of two separate complexes, A and B. IFT Complex A from Chlamydomonas reinhardtii is composed of six subunits: IFT43, IFT121, IFT122, IFT139, IFT140 and IFT144. To explore possible interactions among these subunits yeast two-hybrid analysis was performed. A mix of yeast cells expressing all Complex A subunits linked to the AD domain, was mated with a mix of yeast cells expressing all Complex A subunits linked to the BD domain. Colonies that grew on selective media were analyzed by PCR to determine which subunits were interacting. A strong interaction was observed between IFT 43 and IFT121. Subsequent two-hybrid analysis by co-transformation confirmed the specificity of the interaction; deletion analysis revealed that the C-terminal portion of IFT 121 was sufficient for interaction with IFT43. Bacterial co-expression in E. coli resulted in the same results as the yeast two-hybrid analysis. Isolation and characterization of the interacting proteins in a eukaryotic system has been facilitated by the construction of yeast expression plasmids which allow us to tag each subunit with a unique epitope. The use of Maltose Binding Protein (MBP) as a tag allows for rapid and specific purification of the interacting oligomeric IFT subunits and subsequent analysis. Supported by NIH GM61920 and P20RR016454.

1032/B240

Chlamydomonas DYF-1 is an IFT Particle Complex B Subunit Required for Flagellar Assembly.

Z. Wang, S. Williamson, H. Zhang, D. Liu, H. Qin; Biology Department, TAMU, College Station, TX

The assembly and maintenance of cilia/flagella is dependent on the motility process of intrafagellar transport (IFT). IFT involves large protein particles moving between flagellar base and tip. DYF-1 is originally identified as an activator for the anterograde IFT motor OSM-3 kinesin, specifically required for OSM-3 to dock onto and move IFT particles in Caenorhabditis elegans. However, the ubiquity of the role of DYF-1 functioning as OSM-3 activator in IFT is questionable since many ciliates do not contain an osm-3 homologue but have a dyf-1 gene in their genomes. Moreover, Zebrafish fleer (homologue of dyf-1) shows a pleiotropic defect in ciliogenesis, whereas the role of Kif17/OSM-3 in zebrafish ciliogenesis appears to be cell type specific. Kif17 is essential for photoreceptor cell outer segment formation, but largely dispensible for ciliogenesis in general. Therefore, to investigate the relationship between DYF-1 and IFT, we have characterized a DYF-1 homologue (CrDYF-1) in Chlamydomonas reinhardtii, which has no OSM-3 homologue in its genome. Through biochemical analyses, we show that CrDYF-1 is a bona-fide IFT particle complex B protein, which is present in a molar ratio of ~1 to other components of the IFT particle subunits. CrDYF-1 accumulates at basal bodies and localizes along the flagella as punctual dots. Cells with a reduced level of CrDYF-1 by RNA interference are defective in flagellar assembly, indicating CrDYF-1 is essential for flagellar assembly.

1033/B241

A Role for CaMKII-mediated Phosphorylation of Kif17 in Photoreceptor IFT.

C. Insinna, M. Humby, J. Besharse; Cell Biology, Neurobiology& Anatomy, Medical College of Wisconsin, Milwaukee, WI

Vertebrate photoreceptors are sensory neurons that require intraflagellar transport (IFT) to move cargo in and out of a modified cilium called the outer segment (OS). We have recently shown that in addition to the canonical kinesin II motor a second homodimeric kinesin called Kif17 also plays a prominent role in photoreceptor IFT (Insinna, et al., Dev. Biol., 2008). Recently, the phosphorylation of Kif17 by calcium-calmodulin-dependent protein kinase II (CaMKII) was shown to act as a molecular switch that regulates vesicle delivery in neurons (Guillaud, Nat. Cell. Biol,. 2008). Here we have examined the idea that inhibition of CaMKII-mediated phosphorylation of Kif17 would alter photoreceptor IFT. Antibodies for IFT complex A and B proteins were used in immunoprecipitation (IP) and immunocytochemical (IC)

assays on mouse retinae treated with either a CaMKII inhibitor or peptides that compete for the CaMKII binding site of Kif17. Additionally, Kif17 dominant negative and constitutively active CaMKII were expressed specifically in zebrafish cone photoreceptors and analyzed by fluorescence and transmission electron microscopy. Our data show that inhibition of CaMKII dissociates Kif17 from IFT proteins. Furthermore, overexpression of constitutively active CaMKII in photoreceptors disrupts disc assembly within the OS. Our results suggest that CaMKII phosphorylation of Kif17 regulates entry of Kif17 and other IFT components into the OS.

1034/B242

Sensory Cilium Distal Segment Assembly: Regulators of the IFT Motor, OSM-3.

L. Hao, B. Prevo, D. Carlson, S. Acar, J. Scholey; Molecular and Cellular Biology, UC Davis, Davis, CA

The assembly of the distal segments of sensory cilia, which play key roles in cilium-based signaling, depends upon the action of homodimeric kinesin-2, OSM-3/KIF17, in C. elegans chemosensory neurons and Zebra fish retina. The C. elegans OSM-3 motor, which drives the fast anterograde transport of IFT particles all along the cilium, is proposed to exist in an autoinhibited state which might be activated by components of the IFT-particles. In genetic screens for components of the distal segment assembly pathway, we found two genes, qj55 and qj23 that phenocopy the osm-3 mutants in assays of IFT, dye-filling and cilium morphology. Complementation tests and SNP mapping reveal they are new alleles of dyf-6 and ift-81. In dyf-6(qj55), all IFT proteins tested, including OSM-3::GFP, move at the slow rate characteristic of heterotrimeric kinesin II, suggesting that OSM-3 is inactive but is moved passively in the autoinhibited state. This is supported by analysis of osm-3::GFP and dyf-1::GFP markers in double mutants of dyf-6(qj55) with klp-11(kinesin II) or bbs-8(BBS). We conclude that DYF-6 is an activator of OSM-3 motility. In ift-81(qj23), all IFT markers move at the slow, kinesin-II rate, except for OSM-3::GFP, which moves at a composite OSM-3/kinesin-II rate. By analyzing double mutants of ift-81 with klp-11(kinesin II) or bbs-8(BBS) with osm-3::GFP and dyf-1::GFP markers, we obtained evidence that OSM-3 is not fully functional. Additional assays using yeast 2 hybrid experiments and electron microscopy are being used to determine the mechanism by which DYF-6 and IFT-81 control the OSM-3 dependent assembly of ciliary distal segments and thereby contribute to cilium-based signaling.

1035/B243

G Protein Signaling and the GMAP210 Homologue SQL-1 Modulate Intraflagellar Transport in C. elegans.

J. Broekhuis, S. Rademakers, M. Dekkers, J. Burghoorn, G. Jansen; Cell Biology, Erasmus MC, Rotterdam, Netherlands

Recent studies suggest that the localization of signaling molecules in the cilia and the structure of cilia are regulated by the intraflagellar transport (IFT) machinery. In *C. elegans* this regulation may be achieved by the coordination of two kinesin-2 motor complexes used for anterograde IFT, kinesin II and OSM-3. In the cilia middle segments OSM-3 and kinesin II move together at a speed of 0.7 µm/s, and in the distal segments OSM-3 moves alone at 1.2 µm/s. In *osm-3* mutants kinesin II moves at 0.5 µm/s in the middle segments. We found that in both loss- (lf) and gain-of-function (*gpa-3QL*) mutants of the heterotrimeric Gα *gpa-3* OSM-3 moves at 1 µm/s and kinesin II moves at 0.6 µm/s, while complex A and B particle proteins move at 0.75 µm/s. This suggests that the cilia of *gpa-3* mutant animals contain three types of IFT particles: particles transported by OSM-3 or kinesin II alone, and perhaps a small subset transported by both kinesins. Interestingly, *gpa-3QL* results in shorter cilia, while *gpa-3(lf)* does not affect cilia morphology. We propose a model in which GPA-3 regulated docking of either kinesin II, OSM-3 or both, determines entry of IFT particles into the cilia subdomains. This mechanism would allow plasticity of cilia structure and function. We performed a genetic screen for suppressors of *gpa-3QL* and identified *sql-1*, which encodes the homologue of the mammalian Golgi protein GMAP-210. *sql-1(lf)* suppresses the effect of *gpa-3QL* on cilia length, but does not seem to affect cilia morphology by itself. In *sql-1(lf)* animals OSM-3 moves faster and kinesin II moves slower, suggesting that the two kinesins are at least partially uncoupled. However, both complex A and B proteins move at the same speed as OSM-3. This suggests that in *sql-1(lf)* animals IFT is predominantly mediated by OSM-3 kinesin, which would explain the suppression of the effect of *gpa-3QL* on cilia length.

1036/B244

Primary Cilia Function in Shh Producing and Responding Cells of the Limb Bud.

A. K. O'Connor[1], C. J. Haycraft[3], N. F. Berbari[1], J. Verheyden[2], X. Sun[2], B. K. Yoder[1]; [1]Cell Biology, University of Alabama at Birmingham, Birmingham, AL, [2]Genetics, University of Wisconsin - Madison, Madison, WI, [3]Medicine, Medical University of South Carolina, Charleston, SC

Primary cilia are small protrusions found on most cells of the mammalian body which are formed by a process called intraflagellar transport (IFT). Disruption of ciliogenesis through mutations in the IFT proteins IFT88 (Polaris) or Kif3a (IFT kinesin) result in early embryonic lethality. In vivo and in vitro analyses of the phenotypes seen in cilia mutants indicate that this organelle has a critical role in regulating several signaling pathways, including hedgehog and wnt, both of which are important for normal development, differentiation, and fate determination. Using IFT88 mutant limb bud cultures we previously found that cells lacking cilia are unable to respond to sonic hedgehog (Shh) and have defects in activation and processing of the hedgehog responsive transcriptional regulators Gli2 and Gli3, respectively. In addition, many of the Shh pathway components including Patched, Smoothened, and the Gli proteins, have been shown to localize to the cilium. To further analyze the function of cilia in Shh signal transduction and development, we are using conditional alleles of these two ciliogenic genes and tamoxifen inducible transgenic CreER mouse lines to disrupt cilia in Shh producing or responding cells. Our initial analysis of mice where cilia are ablated on Shh producing cells throughout development reveal there is a marked truncation of the posterior two digits, those derived from Shh producing cells and lacking cilia. This is in contrast to the extensive polydactyly seen mice lacking cilia on the cells throughout the limb mesenchyme. Analyses with the Shh responding cells are currently underway. Using this inducible system we are selectively eliminating cilia at specific timepoints during development, and in specific cell types, allowing us to delineate the role that cilia signaling activities have in normal embryogenesis and cell fate determination.

1037/B245

GEFs in Ciliary Evolution.

P. Satir, A. Awan, A. Bell; Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Guanine nucleotide exchange factors (GEFs) are proteins that activate small G proteins by exchanging GTP for bound GDP. Classes of GEFs include the Arf Sec7 GEFs, several members of which are found in ciliates. Nair et al. (1999) identified a Paramecium Sec7 GEF (PSec7) in the ciliome and developed a PSec7 Ab that localizes along somatic and oral cilia and to the macronucleus in both Paramecium and Tetrahymena. Molecular analysis identifies the probable Tetrahymena protein as GEF1, a 238 kDa molecule with ciliary and nuclear targeting sequences. GEF1 is transported into the cilium by an IFT homodimeric kinesin, Kin 5, to become a resident ciliary protein. A tagged truncated GEF1 with cilia but not nuclear localization signals is found at the cilium tip. We hypothesize that the GEF1 functions to activate an Arf- like G protein that in turn acts on IFT complexes to release cargo, perhaps specific ciliary membrane receptors or channels. This mechanism can be generalized to GEF/G-protein pairs, found in metazoan motile and primary cilia, which when disrupted result in ciliopathies. It could also be evolutionarily related to the mechanisms of nuclear import that require a Ran GEF (e.g. RCC1) resident in the nucleus.

1038/B246

The Role of Second Messenger Signaling in Primary Cilium Length Regulation: Implications for Flow-Mediated Adaptation.

T. Y. Besschetnova[1,2], B. Roy[2], J. V. Shah[1,2,3]; [1]Department of Systems Biology, Harvard medical School, Boston, MA, [2]Department of Medicine, Renal Division, Brigham and Women's Hospital, Boston, MA, [3]Harvard-MIT Division of Health Sciences and Technology, Boston, MA

A host of studies of the primary cilium, an antenna-like structure extending from the cell surface, has shown that dysfunction of this organelle is implicated in a variety of disease states of which almost all manifest polycystic kidneys. It is believed that in environments such as the lumen of the renal tubule the primary cilium transduces extracellular cues to maintain tissue homeostasis. Cilium length directly impacts the signaling strength and detection sensitivity of these extracellular cues. To explore this cilium length control in mammalian cells we evaluated cilium length dynamics and intraflagellar transport (IFT) in mouse Inner Medullary Collecting Duct cell lines under pharmacological of modulation major cell signaling pathways. Effectors of Ca2+, cAMP, cGMP, and GSK3 signaling were used to study the regulation of cilium length. Our findings show that treatment of cells with GSK3 inhibitors, Ca2+ blockers and agonists of cAMP signaling led to a dramatic and rapid (3 hours) increase in cilium length of ~2-3-fold. RNAi-mediated knockdown of polycystin-2 (PC2), a genetic cause of autosomal dominant polycystic kidney disease (PKD), completely blocked the length increase caused by GSK3 inhibition and Ca2+ blockade and partially suppressed the cAMP-induced lengthening. Furthermore, application of fluid flow, and downstream activation of cilium-based signaling, partially suppressed the effect of cAMP activation, but not Ca2+ blockade. Epistatic relationships uncovered by dual pharmacological and RNAi-mediated gene knockdown have revealed the signaling logic of this pathway. The cilium length control pathway itself must also impinge on the intraflagellar transport machinery itself. We observed strong correlation between cilium lengthening and the increase of anterograde (~2X increase) transport velocities of IFT88-EYFP particles measured in live primary cilia by high-resolution timelapse microscopy. Together these data demonstrate that signaling can regulate cilium length through the regulation of IFT. The role of PC2 in this adaptation may implicate cilium length regulation in the pathogenesis of PKD.

1039/B247

Deregulation of IFT Particle Distribution on RNAi of Trypanosoma brucei NPHP3-like.

H. Dawe, T. Barge, H. Farr, E. Browne, K. Gull; Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

Eukaryotic cilia and flagella are highly conserved structures generally composed of a canonical 9+2 microtubule axoneme. Impairment of cilia and flagella function underlies a growing number of human genetic diseases. However, the functions of many gene products underlying these disorders are unknown; consequently treatment remains challenging. Inducible RNAi in the flagellate parasitic protozoan *Trypanosoma brucei* is a useful tool for analysing gene function. We performed RNA interference knockdown experiments of a novel trypanosome TPR-repeat-containing protein that bears similarity to the juvenile nephronophthisis protein NPHP3. We found a motility phenotype and ultrastructural defects consistent with a flagellar role for this protein. A full-length flagellum was built, but in common with many trypanosome flagellar mutants the two central pair microtubules were mis-oriented. However, most importantly we observed an unusual increase in the number of intraflagellar transport (IFT) particles in the mutant, particularly at the base of the flagellum near the transitional fibres. These are thought to be the site of docking of IFT complexes, and in contrast to wild-type cells, at this region in the mutant each doublet microtubule was associated with an IFT particle, suggesting deregulation of IFT.

1040/B248

A Homologue of Joubert Syndrome-Associated Arl13b Regulates Anterograde Intraflagellar Transport in C. elegans.

S. Cevik, O. Blacque; University College Dublin, Dublin, Ireland

Cilia biogenesis, morphological shaping and function require intraflagellar transport (IFT), an evolutionarily conserved and cycling bidirectional motility of protein complexes along ciliary axonemes. Numbering over 20 proteins, organised into kinesin 2, subcomplex A, subcomplex B and dynein modules, the IFT machinery traffics cargos required for building and maintaining cilia. Defects in cilia function contribute to various human diseases including primary ciliary dyskinesia, cystic kidney disease and retinal degeneration, as well as pleiotropic syndromes such as Bardet-Biedl syndrome, Meckel syndrome and Joubert syndrome. Although many IFT components have been identified, how IFT is regulated remains poorly understood. Using the *C. elegans* sensory cilia model, we identify ARL-13 (ADP-ribosylation-like factor 13), a homologue of Joubert syndrome-associated Arl13b, as a regulator of anterograde IFT. First, we show that ARL-13 localises predominantly to ciliary middle segments and provide evidence that ARL-13 is membrane-associated via posttranslational modification. Next, we find that *arl-13* gene mutants possess defects in function and morphology, including ultrastructural defects. Using *in vivo* IFT motility assays, we subsequently observed that anterograde kinesin 2 motors (OSM-3-kinesin and kinesin-II) and subcomplex A/B components are abnormally uncoupled and moving independently along *arl-13* mutant cilia. In addition, the rate of OSM-3 translocation is retarded. Taken together, we propose that ciliary membrane-associated ARL-13 coordinates the assembly of anterograde IFT complexes and suggest that defects in this function underlie the molecular aetiology of Arl13b-associated Joubert syndrome.

1041/B249

The Small GTPase Ran and Nuclear Transport Adapter Protein Importin-alpha Are Present in the Flagella in Chlamydomonas reinhardtii.

C. Tsao, C. R. Wood, J. Rosenbaum; Mol. Cell. Dev. Biol., Yale University, New Haven, CT

Protein targeting and trafficking between compartments is important for organelles to perform their specific functions. To transport proteins into the nucleus, the nuclear trafficking receptor, importin-beta, binds cargo directly, or indirectly through an adapter protein, importin-alpha, which in turn binds the nuclear localization signal on the cargo. The directionality of transport is regulated by the small GTPase Ran. Ran has also been found to be critical for other cellular processes, including assembly of the mitotic spindle and nuclear envelope. A proteomic analysis suggested that importin-alpha and Ran are present in the flagella of the biflagellate alga *Chlamydomonas reinhardtii* (Pazour et al. 2005. *J. Cell Biol.* **170**:103-113). In this work, we generated antibodies against the *Chlamydomonas* importin-alpha (CrIPA) and Ran (CrRan) orthologs, which share ~60% and ~78% amino acid sequence identity with their mammalian homologs, respectively. Immunoblot analyses verified the presence of CrRan and CrIPA in the soluble matrix fraction of *Chlamydomonas* flagella. Using immunofluorescence microscopy, we observed that both CrRan and CrIPA are localized in a punctate pattern along the flagella. Regulation of the passage of proteins to and from the flagellar compartment and the mechanism of loading and unloading of cargo transported along the flagellum by IFT are two important areas of research for which little data has been accumulated. Current models for how Ran modulates cellular processes center on the concept that proteins exist in inhibitory complexes that are relieved by Ran binding, making Ran an attractive candidate for a regulator of cargo release during flagellar protein transport events. It has been hypothesized that a region at the junction of the basal body and the flagellar axoneme may function as a regulatory pore, controlling which molecules pass into or out of the flagellum (Bloodgood, R. 2000. *Cell Biol. Internat.* **24**(21):857-862). The flagellar localization Ran and importin-alpha, two proteins central to the trafficking of proteins across the nuclear pore, may represent a foot in the door leading to the characterization of a flagellar pore system. This work is supported by NIH grant GM14642.

1042/B250

IFT Particles Participate in Flagellar Sexual Signaling in Chlamydomonas, but Membrane Protein Trafficking into Flagella Is IFT-Independent.

W. J. Snell, C. Hernandez, Q. Wang; Cell Biology, UT Southwestern Medical Center at Dallas, Dallas, TX

Cilia and flagella are key signaling organelles and their axonemal core is assembled by the intraflagellar transport (IFT) machinery. Our laboratory is using biochemical and genetic methods to study the role of IFT in signaling and in transport of membrane proteins from the cell body to the flagella in Chlamydomonas. Previously, we showed that IFT particles form large assemblies during the cilium-generated, protein kinase-dependent signaling that occurs when mt+ and mt- gametes adhere to each other by gamete-specific flagellar adhesion molecules, the mt+ and mt- agglutinins. In kinesin-2 mutant gametes placed at 32C, the anterograde IFT motor and IFT particles are lost from the flagella after ~45 min and the flagella remain structurally intact and motile for 1-2 hrs. Moreover, at 32C the mutant gametes undergo normal flagellar adhesion, but cAMP formation is abrogated, and the cells disaggregate because the lost agglutinins are not replaced by transport of cell body agglutinins to the flagella. Here, in continued studies on IFT function during flagellar adhesion, we show that IFT particles undergo additional adhesion-triggered changes. The amounts of IFT particle within flagella are increased several fold and the IFT subcomplex A particle protein 139 is altered. A more slowly migrating form of IFT139, which is present only in flagella and interacts with the major flagellar membrane glycoprotein, is lost or converted to the more rapidly migrating form. We also have extended our previous finding of IFT-independent movement of agglutinins onto flagella. We show that when wt gametes are mixed with kinesin-2 mutant gametes of the opposite mating type at the non-permissive temperature, only the kinesin-2 gamete flagella are depleted of agglutinins. And, when db-cAMP is added, the kinesin-2 mutant gametes re-acquire their flagellar agglutinins. Thus, our results show that IFT is essential for cilium-generated signaling, and that IFT particles organize and participate directly in signaling. IFT, however, participates only in signal generation and is not required for transport of the agglutinin membrane protein from the cell body to the flagella.

1043/B251

Purification of Intact Flagella of Trypanosoma brucei And Analysis of Novel Matrix and Membrane Components.

D. Julkowska, P. Bastin; Pasteur Institute, Paris, France

Trypanosoma brucei is a flagellated protist responsible for sleeping sickness in Africa but also an excellent model organism to study flagellum formation. Presently little is known about the control of the synthesis of intraflagellar transport (IFT) proteins as well as the mechanisms by which IFT particles are assembled, cargo loading and release or regulation of motor activity or motor exchange at the base and the tip of of the flagellum. We decided to purify intact flagella of *T.brucei* and to determine protein components of flagellar matrix and membrane fraction with a particular attention to possible sensing proteins. Until now, adhesion of the flagellum to the cell body prevented its purification in intact form, required the use of strong detergents that destroyed flagellar membrane and allowed purification of cytoskeletal fraction only. We used the *FLA1*(a surface glycoprotein required for the adhesion of the flagellum) RNAi mutant in which the flagellum is detached from the cell body but its formation process and structure remain normal. We developed a new deflagellation method, based on mechanical detachment, that allows isolation of intact flagella from FLA1. The integrity of the flagellar structure was confirmed by scanning electron microscopy as well as by immunofluorescence assay against known membrane and matrix flagellar proteins. The composition of the flagellar cytoskeleton and soluble fractions was analyzed using mass spectrometry and resulted in identification of 265 proteins including already known flagellar components as well as 75 hypothetical proteins. These are now being studied in terms of their localization in the flagellum (GFP fusion), function (RNAi mutants) and protein-protein interactions (Tap-Tag, immuno-precipitation).

1044/B252

Primary Cilia Regulate Branching Morphogenesis and Mammary Gland Lobular-Alveolar Development.

K. M. McDermott[1], T. Tlsty[1], G. Pazour[2]; [1]Pathology, University of California San Francisco, San Francisco, CA, [2]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA

The mammary gland is composed of tubes arising from an epithelial bud that expands during development into a continuous tree-like network of branched ducts via a process known as branching morphogenesis. Primary cilia project from the surface of cells and act as antennae to mediate signal transduction pathways for communication with the environment. Previous electron microscopy studies reported the presence of primary cilia on myoepithelial cells in mammary glands of diverse species; however, there have been no studies to determine if cilia play a role in mammary gland development and function. We found that cilia are present on myoepithelial and stromal cells throughout developmental and functional (pregnancy and lactation) stages. In contrast to published reports, we found that cilia are present on the luminal epithelial cells during early stages of development, but the numbers decreased significantly by late development. Thus, cilia project from all three major cell types of the organ and are ideally positioned to regulate development and function of the gland. We address the function of primary cilia in mammary tissue by analyzing the pathology that results when cilia are defective due to a mutation in intraflagellar transport (Tg737oprk mouse model). We found that ciliary dysfunction results in decreased ductal invasion and a decrease in branching during mammary gland development. Furthermore, we have demonstrated that primary cilia play a role in branching morphogenesis and lobular-alveolar development during pregnancy and lactation. To our knowledge, this is the first report to reveal a role for primary cilia in branching morphogenesis and lobular-alveolar development. More studies will also be necessary to fully resolve the mechanism(s) by which primary cilia control these processes. Nonetheless, our studies uncover a previously unknown function for primary cilia and further highlight their significance in developmental biology.

Muscle: Biochemistry & Cell Biology (1045 – 1067)

1045/B253

Myofibril Assembly Visualized by Imaging N-RAP, Alpha-Actinin, and Actin in Living Cardiomyocytes.

S. M. Manisastry, K. Zaal, R. Horowits; NIAMS, National Institutes of Health, Bethesda, MD

N-RAP is a striated muscle-specific scaffolding protein found in developing myofibrils. Previous studies suggest that N-RAP organizes alpha-actinin and actin into symetrical I-Z-I structures, with the barbed ends of actin filaments anchored at a central Z-body or Z-line. The present study aims to understand the order of events during myofibril assembly through time-lapse confocal microscopy of cultured embryonic cardiomyocytes expressing fluorescently-tagged proteins. Primary cardiomyocyte cultures were prepared from day 7 chick embryos and double transfected with EYFP-alpha-actinin or EYFP-actin (generous gifts from Dr. Joseph Sanger, SUNY Upstate Medical University) and mCherry-NRAP plasmids. During de novo myofibril assembly, N-RAP assembled in fibrillar structures within the cell, with dots of alpha-actinin subsequently organizing along these structures. The initial fibrillar structures were reminiscent of actin fibrils, and this was supported by simultaneous coassembly of N-RAP and actin into newly formed fibrils. The alpha-actinin dots were often initially organized along the N-RAP fibrils with periodic longitudinal spacings of 1.5-2.0 µm, similar to the sarcomere spacing in mature myofibrils, demonstrating that periodicity is established in the premyofibril before or simultaneously with alpha-actinin recruitment, possibly by N-RAP interaction with actin. The alpha-actinin dots subsequently broadened to Z-lines that were wider than the underlying N-RAP fibril, and N-RAP fluorescence intensity decreased. FRAP experiments showed that most of the alpha-actinin dynamically exchanged during all stages of myofibril assembly. In contrast, less than 20% of the N-RAP in premyofibrils was exhanged during 10-20 minutes after photobleaching, but this value increased to 70% during myofibril maturation. The results show that N-RAP assembles into an actin containing scaffold before alpha-actinin recruitment; that the N-RAP scaffold is much more stable than the assembling structural components; that N-RAP dynamics increase as assembly progresses; and that N-RAP leaves the structure after assembly is complete.

1046/B254

Enhancement of Cardiac Myogenesis and Contractility by Overexpressing TNNI3K, A Novel Cardiac Troponin I-Interacting Kinase.

Z. Lai[1], Y. Chen[2], X. Meng[3], J. Ding[3], P. Li[4], X. Cheng[4], Y. Kitamoto[5], S. Kim-Mitsuyama[1]; [1]Department of Pharmacology and Molecular Therapeutics, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan, [2]Department of Immunogenesis, Graduate School of Medical Sciences, Kumamoto University, Kumamoto, Japan, [3]Cardiovascular Institute and Fuwai Hospital, PUMC & CAMS, Beijing, China, [4]The 2nd Hospital and Institute of Cardiovascular Disease Research, Nanchang University, Nanchang, China, [5]Department of Internal Medicine, Sendai Shakaihoken Hospital, Sendai, Japan

Background: TNNI3K is a novel cardiac-specific and troponin I-interacting MAP kinase. In this study, we investigated the effects of TNNI3K on in vitro cardiomyogenesis and on in vivo ischemic injury in mice. Methods and Results: In a cardiac myogenesis model, pluripotent P19CL6 cells transfected with pcDNA6-TNNI3K plasmid were induced to differentiated beating cardiomyocytes by incubating cells in the medium containing 1% dimethylsulfoxide for 4 days. TNNI3K-overexpression promoted cardiomyogenesis by increasing the number of beating masses and α-actinin-positive cells. Contractile force and beating frequency of the cells also increased in the TNNI3K overexpressing cells as compared with the vector-only transfected cells($P<0.05$). In single cardiomyocytes isolated from the beating masses, TNNI3K increased the velocity of diastolic depolarization, prolonged action potential duration($P<0.05$) and enhanced epinephrine response of spontaneous action potentials. TNNI3K also suppressed phosphorylation of cardiac troponin I, suppressed Bax, p38, JNK or ERK-mediated apoptosis, and decreased the number of annexin-V+ cells. In a myocardial infarction(MI)model of mice, intramyocardial injection of P19CL6 cells overexpressing TNNI3K attenuated ventricular remodeling when compared with injection of P19CL6 cells transfected with vector-only. Transthoracic ultrasound cardiography before MI and 2 weeks after MI showed improvement of impaired ejection fraction, left ventricular end-diastolic and end-systolic dimensions. Histological examination revealed that infarct size was significantly smaller in the TNNI3K-overexpressing group than the medium-only or vector-only groups ($P<0.05$). The occurrence of TUNEL-positive cells in the infarct border zone was significantly lower in the TNNI3K-overexpressing mice ($p<0.05$). Conclusions: Our study clearly indicates that TNNI3K promotes cardiomyogenesis, enhances the cardiac performance and protects the heart from ischemic injury through suppressing apoptosis and the phosphorylation of cardiac troponin I. Therefore, modulation of TNNI3K activity would be a useful therapeutic approach for ischemic cardiac disease (This work was supported by JSPS Japan-China Medical Exchange Program).

1047/B255

Tropomyosin is Required for Cardiac Myofibril Assembly, Cardiac Development, and Viability in the Mouse.

C. McKeown, V. Fowler; Department of Cell Biology, The Scripps Research Institute, La Jolla, CA

Tropomyosins are actin-binding proteins that play two key roles in muscle: they act in muscle contraction by regulating the actin-myosin interaction via troponins, and they stabilize the thin filament by binding along its length. While much is known about the regulatory role of tropomyosins in contraction, little is understood about the role of tropomyosin in stabilizing the thin filament during myofibril assembly. We are interested in testing the contribution of tropomyosin to myofibril assembly during cardiac development in vivo. To this end, we have begun analyzing the consequences of a targeted deletion in the mouse Tpm1 gene that targets the alpha-tropomyosin 1 (αTM1) isoform. During development, the embryonic mouse heart predominantly expresses αTM1 and mice lacking αTM1 are embryonic lethal at E9.5-E10. Embryonic development ceases during the cardiac looping stage, suggesting that αTM1 is required for looping morphogenesis. By whole-mount immunofluorescence, αTM1 null cardiomyocytes do not make mature myofibrils but instead display bundles of actin filaments with periodic puncta of alpha-actinin reminiscent of nascent myofibrils. Moreover, tropomodulin1 is associated with the actin filaments within these nascent myofibrils, suggesting that the actin filaments are capped by tropomodulin1 despite being misaligned and not organized into mature sarcomeres with defined H zones. Staining for both sarcomeric myosin and myomesin indicates that these thick filament components are concentrated in the nascent myofibrils in the αTM1 null hearts, but are not assembled into organized and striated thick filament structures. These data suggest that αTM1 is required for maturation of nascent myofibrils to striated myofibrils in vivo. Furthermore, myofibril assembly may be necessary for cardiac looping and the progression of cardiac development in the mouse.

1048/B256

Rac1-mediated β-actin Dynamics in Hypertrophying Adult Cardiomyocytes.

S. Balasubramanian, S. Mani, D. Kuppuswamy; Medicine, MUSC, Charleston, SC

Objective: Adult cardiomyocytes respond to hemodynamic overloads by increasing their size. The cardiomyocyte cytoskeletal attributes responsible for this process are not fully understood. While α-actin in cardiomyocytes governs muscle contraction, the role of β-actin is yet to be studied. We intended to test β-actin dynamics in response to hypertrophic stimuli and the role of Rac1, a regulator of actin cytoskeleton, in this process. Methods: We 1) adenovirally expressed GFP-β-actin in adult cardiomyocytes and followed the fluorescence recovery after photobleaching (FRAP) to measure actin dynamics at various cellular locations upon hypertrophic stimulation, 2) measured the distribution of β-actin into filamentous (F) and globular (G) fractions in hypertrophic myocardium in vivo and agonist stimulated adult cardiomyocytes in vitro, and 3) determined the role of Rac1 in β-actin dynamics in adult cardiomyocytes by using both dominant negative Rac (N17Rac) adenovirus and a pharmacological Rac inhibitor, NSC23766. Results: β-actin is expressed in intact adult myocardium and is distributed at Z-discs and costameres. During pressure overload-induced hypertrophy in vivo, β-actin and Rac1 are associated with Triton-insoluble cytoskeletal and membrane skeletal complexes. Hypertrophic induction in vivo and in vitro increased the amount of β-actin in the F fraction, and these changes were accompanied by the phosphorylation of cofilin and LIMK. Expression of GFP-β-actin in adult cardiomyocytes exhibited its localization at Z-discs, costameres and cell termini. FRAP studies demonstrate dynamic assembly of β-actin into costameres, Z-discs and cell termini. Blocking Rac1 1) blocked agonist-induced Rac1 recruitment to the membrane skeleton and cofilin phosphorylation, 2) caused accumulation of β-actin into intracellular punctate structures and 3) greatly reduced the rate of reassembly of GFP-β-actin in adult cardiomyocytes. Conclusion: These studies demostrate for the first time Rac1-mediated β-actin dynamics in cardiomyocytes suggesting its importance in cardiac cytoskeletal rearrangement during hypertrophy.

1049/B257

Association of Hsp27 With Myofibrils in Injured Cardiac Muscle Is Independent of Filamentous Actin.

N. R. Tucker, E. A. Shelden; School of Molecular Biosciences, Washington State University, Pullman, WA

Injury to muscle tissue plays a central role in acute cardiac ischemia and multiple muscle disorders. Overexpression of Hsp27 has been shown to protect muscle cells against thermal, oxidative and ischemic injury. However, underlying mechanisms of this protection are unclear. A distinctive feature of muscle cells is the constitutive and stress induced interaction of Hsp27 with the sarcomere, the primary component of the contractile apparatus in striated muscle tissue. The interaction of Hsp27 with the cytoskeleton in both muscle and non-muscle cells has been widely reported and is thought to represent interaction with filamentous actin. Here, we have examined the association of endogenous Hsp27 with the sarcomeric architecture in adult zebrafish cardiac muscle subjected to both thermal injury (heat shock) and mechanical stretching. Similar to data from mammalian cell systems, heat shock induces translocation of Hsp27 from the soluble fraction to a Triton-X 100 insoluble fraction, and from a cytosolic location to discrete bands within myofibrils. We also conducted a quantitative analysis of the location of Hsp27, desmin, alpha actinin, filamentous actin and titin in heat shocked cardiac muscle tissues under relaxed conditions and after varying extents of myofibril stretching. Results of this analysis reveal that the location of Hsp27 is independent of desmin, alpha-actinin, and filamentous actin in stretched myofibrils. In contrast, Hsp27 bands maintain a consistent relationship with the position of the titin A/I band boundary over various sarcomeric lengths, as revealed by a monoclonal antibody raised against this region. Finally, preferential extraction of F-actin by chaotropic agents followed by immunolocalization and immunoblotting reveals that Hsp27 is bound to an insoluble component of the remaining sarcomere. Together, these data supports a mechanism of Hsp27 function where interactions with the titin filament system protect myofibrils from stress-induced degradation.

1050/B258

Exploring the Dynamics of Myofibrillogenesis in Living Muscle Cells.

J. Wang, Y. Fan, B. Mittal, D. Dube, J. Sanger, J. W. Sanger; Cell and Developmental Biology, SUNY Upstate Medical University, Syracuse, NY

During the growth and maintenance of myofibrils, sarcomeric molecules must be exchangeable so that myofibrils can respond to developmental and environmental cues. We have used Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Resonance Energy Transfer (FRET) to analyze sarcomeric proteins that were expressed as fusions of Green Fluorescent Protein variants (FP) in cardiac

and skeletal myocytes cultured from embryonic quail muscle. FRAP analysis showed that the recovery of myosin heavy chain (MHC) and myosin light chain (MLC) fluorescence after bleaching occurred uniformly along the length of the A-bands with MLC recovering more quickly than MHC. The recovery of MHC was three times faster in cardiac muscle A-bands than in those in skeletal muscle. Alpha-actinin exchanged faster in the Z-bands in cardiac muscle than in skeletal muscle cells, but no differences in exchange rates were detected in actin, FATZ and myotilin in the two types of muscle. Localization of telethonin to the Z-band was not inhibited by mutating its phosphorylation sites nor did these mutated telethonins have altered dynamics as determined by FRAP. The initial site of actin incorporation occurred at the Z-band, whereas tropomyosin (TM) exchange was uniform along the thin filaments. In comparing premyofibrils and mature myofibrils, we found that the rate of TM exchange was faster along the thin filaments of the premyofibrils than in mature myofibrils in skeletal muscle. We speculate that this is due to the absence of nebulin, a TM-binding protein, in premyofibrils, whereas the addition of nebulin to thin filaments in the mature myofibrils may suppress the dynamics of TM. In cardiomyocytes where nebulin levels are very low, TM exchange rates are the same in premyofibrils and mature myofibrils. When the Z-band protein, FATZ, was labeled at both ends, there was a strong FRET signal in premyofibrils that decreased in mature myofibrils. This corresponds to the time that one of FATZ's binding proteins, telethonin, incorporates into the Z-bands, and suggests that the ends of FATZ move apart at this time. Our results demonstrate that FRAP and FRET are powerful tools to explore sarcomeric changes during myofibrillogenesis.

1051/B259

Assembly and Dynamics of Sarcoplasmic Reticulum Domains in Skeletal Muscle Cells.

D. Rossi, V. Cusimano, F. Benini, S. Lorenzini, V. Sorrentino; Neuroscience, University of Siena, Siena, Italy

The sarcoplasmic reticulum (SR) of skeletal muscle cells is a complex network of tubules and cisternae that share a common lumen delimited by a single continuous membrane. The SR contains longitudinal and junctional domains characterized by distinctive patterns of protein localization, but how SR proteins reach or are retained at these sites is not known. Here we report that the organization of the longitudinal SR is a slow process characterized by temporally distinct patterns of protein localization. In contrast, junctional SR proteins rapidly and synchronously assembled into clusters that later merged into mature triadic junctions, but only after completion of longitudinal SR proteins organization. FRAP experiments indicated that organization of the SR was accompanied by significant changes in the dynamic properties of most longitudinal and junctional proteins. Organization of longitudinal SR proteins appeared to be accompanied by a decrease in their mobiity. In particular, the mobility ank1.5 appeared to be affected by a specific interaction with the obscurin protein localized on the myofibrils, while mobility of the InsP3R1 may result from the interaction with the actin cytoskeleton. On the other hand, organization of the junctional SR was accompanied by a more significant decrease in mobility of junctional proteins, which agrees with the current view that these proteins are assembled in a large multiprotein complex. Deletion analysis of representative junctional proteins identified specific amino acid regions that mediate the localization and/or the stable association of these proteins to the junctional SR domain. In conclusion, our studies indicate that the organization of SR domains results from a process of membrane development accompanied by the establishment of specific interactions between resident SR proteins and intrinsic and extrinsic cues.

1052/B260

The Cdc42 GEF UIG-1 is a Substrate for the Giant Protein Kinase TTN-1 in C. elegans.

D. N. Greene, H. Qadota, G. Benian; Dina Greene, Emory University, Atlanta, GA

The muscles of probably all animals contain giant (>700,000 Da) polypeptides that consist primarily of multiple copies of immunoglobulin (Ig) and fibronectin type III domains, and one or even two protein kinase domains. In various muscles these proteins have several roles. For example, directing assembly of the sarcomere and providing passive elasticity (titin of vertebrates), maintenance of the "catch" state (twitchin in mollusks), and ability of insect flight muscle to beat at high frequencies (projectin of insects). We are studying a similar protein in C. elegans, TTN-1. In an ongoing effort to identify binding partners and substrates for the protein kinase domains of these giants, we have an excellent candidate for the TTN-1 protein kinase. The interacting partner is UIG-1, previously defined as an UNC-112 binding partner with Cdc42 GEF activity located in the dense bodies/I-bands of C. elegans striated muscle. An intragenic deletion of uig-1 displays disorganized myofibrils. Using the yeast 2-hybrid method we have determined which portions of each protein are required for this interaction, and we have confirmed this interaction using an in vitro binding assay. By immunofluorescence microscopy, UIG-1 partially co-localizes with TTN-1. Further, TTN-1 kinase phosphorylates UIG-1 in vitro in regions outside the DH and PH domains. We speculate that phosphorylation of UIG-1 by TTN-1 regulates either (1) the interaction of UIG-1 with UNC-112, (2) UIG-1's localization to dense bodies, or (3) the GEF activity of UIG-1. Whichever is the case, these studies have identified the first substrate for a muscle giant kinase in C. elegans and revealed a function for one of these kinases in sarcomere assembly.

1053/B261

Obscurin and Obsl1 - New Structural Players in the Sarcomeric M-Band.

S. Lange[1], A. Fukuzawa[2], O. Kunfu[1], J. Chen[1], M. Gautel[2]; [1]Department of Medicine, University of California San Diego, La Jolla, CA, [2]Randall and Cardiovascular Divisions, King's College London, London, United Kingdom

The sarcomere in cross striated muscle cells is formed of structural, signaling and scaffolding proteins. While the main function of the sarcomere is to generate contraction by the action of actin and myosin, a large amount of additional proteins are vital for the assembly, support, maintenance and adaptation of heart and skeletal muscle. The objective of this study is to decipher the complex M-band protein-interaction network of the sarcomere using molecular biological, cell biological and genetic approaches. In a screen for binding partners to the M-band proteins myomesin and titin, we were able to identify obscurin-like 1 (Obsl1) and obscurin as potential interaction partners. Obscurin-like 1, a novel M-band protein displays high structural and functional homology to the obscurin N-terminus. Analysis of the interaction showed that the M-band localization of obscurin and obscurin-like 1 relies solely on their association with myomesin and titin. The binding and subcellular localization of the obscurin protein family is disturbed by mutations in titin that are known to cause muscular dystrophy (LGMD2J). Further studies will provide further insights into the function and role of obscurin and obscurin like-1 during development and disease.

1054/B262

Comparative Phosphogenomics of Flightin in Drosophila Flight Muscles Reveal Conserved Basal and Lineage Specific Phosphorylation Sites.

J. Vigoreaux[1], P. Lekkas[1], F. Soto-Adames[1,2], B. A. Ballif[1]; [1]Biology, University of Vermont, Burlington, VT, [2]Illinois Natural History Survey, University of Illinois, Champaign, IL

Phosphorylation is a widespread cellular mechanism for regulating protein function. Our understanding of protein phosphorylation has evolved from one that viewed phosphorylation as a binary on and off switch to one in which phosphorylation modulates multiple functional protein states. The transition from stationary pupa to flying adult in *Drosophila melanogaster* is marked by hyperphosphorylation of flight muscle contractile proteins, namely the thick filament proteins flightin and myosin regulatory light chain, and evidence suggest phosphorylation of each protein occurs in a sequential order. To investigate this, we are using ion trap mass spectrometry to identify phosphorylation sites in eleven flightin isovariants established by two dimensional protein gels. We have identified eight sites (S21, T22, T26, S35, S38, S44, T49, and S139), seven of which are found within a 29 amino acid N-terminal segment that is poorly conserved among drosophilids. The N-terminal region is predicted to be naturally disordered (unstructured) and fast evolving under positive selection, evidence that this region may play distinct functional roles in different *Drosophila* species. Phosphothreonines 26 and 49 are present in all isovariants suggesting they represent basal phosphorylation sites. This is supported by the observation that T26 is conserved or substituted by serine in all 13 species of *Drosophila* examined, while T49 is conserved or substituted by an acidic amino acid. Phosphoserine 44 is found only in the presence of phosphothreonine 49 and phosphothreonine 22 is found only in combination with phosphoserine 21 and phosphothreonine 26. Phosphoserines 35 and 38 are conserved in all species while phosphothreonine 22 is conserved only within the melanogaster group. These and other results suggest that hyperphosphorylation of flightin arises from the action of multiple kinases acting independently and in sequential order. When interpreted in an ecological and phylogenetic framework, our results indicate that phosphorylation of flightin fulfills distinct functional roles. We propose phosphorylation modulates a conserved muscle physiological function and a rapidly evolving behavior-associated function.

1055/B263

Expression of a Rippling Muscle/ Myasthenia Gravis Antigenic Domain of Titin N2-A as a Glutathione-S-Transferase Fusion Protein.

L. Zelinka[2], S. McCann[1], M. Guidos[1], G. Walker[1]; [1]Biological Sciences, Youngstown State University, Youngstown, OH, [2]Biomedical Sciences Program, Kent State University, Kent, OH

Autoimmune immune rippling muscle disease (ARMD) is an autoimmune neuromuscular disorder associated with myasthenia gravis (MG). Rippling muscle disease is identified by percussion or stretch stimulated wave-like muscle contractions. The propagation of these contractions does not involve motor unit action potentials. Previous studies in our laboratory recognized a very high molecular weight skeletal muscle protein antigen identified by ARMD patient antisera was the Titin isoform N2A, ATP synthase 6 and PPP1R3. These previous studies used antisera from ARMD and MG patients as probes in screening a human skeletal muscle cDNA library and several pBluescript clones were shown to support expression of immunoreactive peptides. Current studies in our laboratory have subcloned the immunoreactive domain of titin isoform N2A into pGEX-3X G-S-T fusion vector (G3RMMG6). Sequence analysis of the glutathione-S-transferase/ titin N2A fusion gene indicates the cloned titin domain (GenBank accession # EU428487) is in frame and is derived from a region of N2A spanning the exons 248-to 250, a region that encodes a fibronectin III domain. PCR and EcoR1 restriction mapping studies have shown that the inserted cDNA is of a size that is predicted by the bioinformatics analysis of the subclone. Expression and affinity purification of the fusion protein results in the isolation of a polypeptide of 52 kDal., consistent with what is predicted by the inferred amino acid sequence. Immunoblot analysis of the fusion protein, using rippling muscle/ myasthenia gravis antisera, shows that only the titin domain is immunoreactive. Future studies are planned to use the titin fusion protein in myasthenia graivis survey studies exploring the range of immunoreactivity among this category of patients. Mass spectral studies are also planned to further understand the structure of this specific titin domain.

1056/B264

Quantitative Proteomic Analysis Of Skeletal Muscles Subjected To Exercise.

K. Ojima[1,2,3], H. Sorimachi[1,2]; [1]Enzymatic Regulation for Cell Function, The Metropolitan Institute of Medical Science, Tokyo, Japan, [2]CREST, Japan Science and Technology Agency (JST), Kawaguchi, Japan, [3]Meat Proteins Research Team, National Institute of Livestock and Grassland Science, Tsukuba, Japan

Skeletal muscle is able to accommodate into exercise. However, it is still unclear the molecular adaptations of skeletal muscle with exercise. In this study, we applied mass spectrometry-based analysis (iTRAQ TM) and biochemical assays, to assess quantitative differences in the proteome of tibialis anterior muscles isolated from unexercised wild type mice and exercised wild type mice which were encouraged to run on a treadmill. Our results showed that mass spectrometry analysis allowed the detection of approximately 200 proteins in a buffer-soluble fraction of skeletal muscles. These identified proteins were derived from cytoplasm, mitochondria, nucleus, myofibril, plasma membrane, and so on. An up-regulated pattern was observed for metabolic enzymes and chaperones in exercised muscles. In contrast, calcium binding proteins and myofibrillar proteins were involved in down regulated proteins. These results imply that skeletal muscles response to exercise changed metabolic regulation, calcium homeostasis, and myofibrillar integrity.

1057/B265

Deltex2-Mediated Epigenetic Regulation of Myogneic Differentiation by Histone Methylation Regulating MyoD Expression.

D. Luo[1], V. Natu[1], A. Rustagi[1], T. A. Rando[1,2]; [1]Department of Neurology and Neurological Sciences, Stanford University School of Medicine, Stanford, CA, [2]GRECC and Neurology Service, VA Palo Alto Health Care System, Palo Alto, CA

The regulation of myogenic differentiation is controlled by myogenic regulatory factors which, in turn, are themselves regulated at many levels. Activation of the Notch signaling pathway inhibits myogenic differentiation. We examined the role of the Deltex family of proteins,

originally identified as positive regulators of Notch signaling. Murine Deltex2, but not Deltex1, was expressed in myogenic progenitors and its level correlated inversely with myogenic differentiation. Moreover, Deltex2 was found to inhibit myogenic differentiation by inhibiting MyoD expression albeit in a canonical Notch pathway-independent manner. Rather, inhibition of differentiation was associated with the binding of Deltex2 to key regulatory regions (the "DRR" and "PRR") in the MyoD promoter and the hypermethylation of lysine 9 of Histone H3 at the DRR. These results reveal a novel mechanism by which the Deltex family of proteins regulates cellular differentiation as well as an epigenetic mechanism of the regulation of MyoD expression in postnatal myogenesis.

1058/B266

Neogenin Regulates Skeletal Myofiber Size and FAK and ERK Activities In Vivo and In Vitro.

Y. Yang[2,1], G. Jiang[1], H. Lee[2], J. Kang[2,1], R. Krauss[1]; [1]Developmental and Regenerative Biology, Mount Sinai School of Medicine, New York, NY, [2]School of Medicine, SungKyunKwan University, Suwon, South Korea

Skeletal myogenesis is controlled by myogenic bHLH transcription factors, which cooperate with additional transcription factors to coordinate expression of muscle-specific genes and fusion into multinucleated myofibers. Neogenin, a receptor for the netrin and repulsive guidance molecule (RGM) families of ligands has been implicated in neuronal development, including differentiation and survival of neurons and axon guidance. We have previously shown that neogenin functions as a component of multiprotein complexes found at sites of cell-cell contact that contain several promyogenic proteins, including other related Ig superfamily proteins, Cdo and Boc, and cadherins. Neogenin and its ligand netrin-3 positively regulate myoblast differentiation. Here, we report that mice homozygous for a mutation in the Neo1 gene (encoding neogenin) develop myotomes normally, but have small myofibers at E18.5 and at three weeks of age. Similarly, primary myoblasts derived from Neo1-/- animals fail to form large myotubes in vitro. These defects are associated with low levels of the activated forms of FAK and ERK, and inefficient expression of certain muscle-specific proteins, both in vivo and in vitro. Myoblasts lacking neogenin or Cdo are defective in responding to recombinant netrin. These data suggest that neogenin is required for normal muscle development, perhaps functioning via activation of the FAK and ERK signaling pathways.

1059/B267

c-Abl Regulates Positively Myoblast Differentiation via Interaction with a Promyogenic Receptor Cdo.

B. Kim[1], G. Bae[1], J. Oh[1], Y. Yang[1], H. Lee[1], W. Zhang[2], R. S. Krauss[2], J. Kang[1]; [1]Department of Molecular Cell Biology, Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, South Korea, [2]Department of Developmental and Regenerative Biology, Mount Sinai School of Medicine, New York, NY

Differentiation of skeletal myoblasts into multinucleated myofibers is a multistep process that involves the coordinated activation of a cell-type specific transcriptional program and morphological changes that include elongation, alignment and cell-cell fusion. The ubiquitously expressed non-receptor tyrosine kinase c-Abl has been implicated in signaling events that regulate transcriptional and cytoskeletal processes downstream of cell adhesion-, growth factor receptor- and axon guidance-receptors (Furstoss et al., 2002; Wang, 2004; Zandy & Pendergast, 2008). Therefore, c-Abl is well-positioned to coordinate the changes in both gene expression and cell morphology that characterize cell differentiation. c-Abl has recently been shown to positively regulate myoblast differentiation via shuttling from nucleus to cytoplasm (Puri et al., 2002; di Bari et al., 2006). Here we report that overexpression or knockdown of c-Abl enhances or reduces C2C12 myoblast differentiation, respectively. c-Abl binds to the cytoplasmic tail of Cdo, a promyogenic receptor via its SH3 domain. This interaction appears to be important for the promyogenic function of these molecules. Interestingly, the kinase activity of c-Abl appears to be at least partly dispensable for its promyogenic role. Moreover, knockdown of c-Abl impairs the activation of p38MAPK in differentiating myoblasts and myotubes. Taken together, these data suggest that c-Abl functions positively in myoblast differentiation via activation of p38MAPK triggered by the cell surface receptor Cdo.

1060/B268

Myostatin Reduction in Erythropoietin Application in mdx Dystrophic Mice. A Pilot Trial.

D. Feder[1], A. S. Carvalho[3], S. B. Zyngier[1], J. P. Ferraz[3], L. S. Souza[3], A. C. Santomauro[1], L. P. Oliveira[1], M. R. Ugollini[1], V. P. Lioi[1], L. G. Ferreira[2], M. T. Nunes[2], M. C. Carvalho[2], R. A. dosSantos[2]; [1]Pharmacology, FMABC, Sao Paulo, Brazil, [2]ICB, USP, Sao Paulo, Brazil, [3]Neurology, Santa Marcelina Hospital, Sao Paulo, Brazil

Background: Apart from its erythropoietin function, erythropoietin (Epo) exerts also non-erythroid but tissue protective functions. The potential physiological role of the erythropoietin receptor (Epo-R) in skeletal muscle seems to be the control of proliferation and differentiation of myoblasts and satellites cells. Also, it has been suggested an important role of Epo in response to injury. Material and methods: We analyzed the effects of Epo injection on skeletal muscle of mdx mice to study the effects of long term (12 weeks). Seven mdx mice male received Epo injection of 1000 IU/kg/IP and six mdx mice male received placebo (double blinded, placebo). All animals were submitted to exercise 5 days/week, 20 cm/s, 10 min. The muscle strength was measured weekly for 12 weeks. After 12 weeks of treatment, all mdx mice were undergone to muscle biopsy and muscle samples were collected from right quadriceps and gastrocnemius and stained with acid phosfatase and alkaline phosfatase for histological evaluation. Left gastrocnemius was dissected, frozen in liquid nitrogen, and stored at -80 C. Total tissue RNA were extracted with Trizol Reagent. Total RNA (2 ug) was used for first-strand cDNA synthesis. 200 ng of cDNA was used for Real-time PCR and mRNA of TNF alpha, TGF beta1 and myostatin was measured. Results. The results of this study did not show that 12 weeks of Epo treatment was beneficial for improving muscular strength in mdx mice but improved muscular strength during one week (week seven) in the group taking Epo. The histological analysis did not show differences in necrosis and regeneration between placebo group and group receiving Epo. There were no difference in genic expression of TNF alpha and TGF beta 1 but a significant reduction of myostatin in treated mice. Conclusion: Myostatin reduction in erythropoietin treatment in dystrophic mice could explain some effects of erythropoietin in skeletal muscles. The lack of improvement in muscle strength for more than one week may reflect the amount of Epo dose used or the period of treatment. A higher dose and early use of Epo could be a potential application in our next trial.

1061/B269

Analysis of Allele-Specific Expression of the Mouse ε-Sarcoglycan Gene.

M. Imamura, S. Takeda; Department of Molecular Therapy, National Institute of Neuroscience, NCNP, Tokyo, Japan

ε-Sarcoglycan (SG) was found as a type I transmembrane protein highly homologous to α-SG that is encoded in the causative gene for autosomal recessive limb-girdle muscular dystrophy (LGMD2D). Unlike α-SG, loss-of-function mutations in the ε-SG gene cause autosomal dominant myoclonus-dystonia syndrome (M-D), but not muscular dystrophy. Whereas, upregulation of the ε-SG gene (Sgce) in striated muscle was shown to ameliorate muscular dystrophy of mouse model by compensation of α-SG function. Since Sgce is a maternal imprinting gene, reactivation of the silenced gene might be one of approaches for therapeutic treatment of LGMD2D as well as M-D. Thus, to gain insight into the gene regulation, we analyzed allele-specific expression of Sgce using mice (EtKO) lacking a part of cytoplasmic tail of ε-SG by poly-A trap strategy with a removable exon trap retrovirus vector. Tissue analysis of heterozygous F1 mice (C57BL/6 x EtKO, EtKO x C57BL/6) detected the expression of ε-SG on mRNA and protein levels from only paternal alleles. Weak expression of maternal Sgce in the brain was previously reported, but we did not detect it under our experimental conditions. We further confirmed maintenance of the maternal gene suppression under in vitro circumstances using cultured fibroblast cells. Whereas, treatment of the cultured cells with demethylating agent, 5-azadeoxycytidine, did not release ε-SG expression from maternal imprinting alleles although DNA methylation was found in its promoter region. These results suggested that maternal allele-specific Sgce suppression is strictly maintained by multiple regulation systems. Our Sgce partial knockout mice would be useful for the further study of allele-specific gene regulation of Sgce.

1062/B270

SMTNL-1 Is a Potential Transcriptional Repressor of ER/PR Expression and Mediates Adaptive Responses to Pregnancy in Both Vascular and Striated Muscles.

B. Lontay[1], R. Safi[1], C. N. Fortner[1], S. L. Lengyel[3], K. S. Korach[2], D. P. McDonnell[1], T. A. Haystead[1]; [1]Pharmacology and Cancer Biology, Duke University, Durham, NC, [2]Laboratory of Reproductive and Developmental Toxicology, NIEHS/NIH, Research Triangle Park, NC, [3]Zoology, North Carolina State University, Raleigh, NC

Smoothelin-like protein 1 (SMTNL1) is an early target of the cyclic nucleotide dependent protein kinases PKA and PKG. Studies with smtnl1-/- showed that the protein mediates adaptations in both vascular smooth muscle and striated muscles in response to exercise by regulating myosin phosphatase activity. Female smtnl1-/- mice exhibited greater adaptive changes than male littermates and WT female mice expressed ~50% less SMTNL1 than WT male mice. To investigate the relevance of these differences we studied SMTNL1 expression during pregnancy in WT and smtnl1-/- mice. During pregnancy expression of SMTNL1 increased 4 fold in striated muscle over non-pregnant littermates, peaking at day 15. Decline in SMTNL1 expression correlated with increased expression of ERα (estrogen receptor). This observation suggested a relationship between SMTNL1 expression and sex hormone receptors regulating pregnancy. Investigation of expression of steroid hormone receptors in several tissues showed increased expression of ERα, PR and AR in both male and female smtnl1-/-mice. Strikingly, in detailed fiber typing studies in striated muscle, we found exclusive expression of ERα in type IIb fibers in smtnl1-/- mice during pregnancy and increased expression of the myosin phosphatase subunit MYPT. Pregnancy also induced the expression of type 11b myosin with concomitant increased numbers of type IIb fibers. Our studies suggested an inverse relationship between ER/PR expression compared with SMTNL1 and suggest the protein is a negative regulator of these hormone receptors in vivo. Phosphorylation of SMTNL1 at S301 in response to agonists acting through PKA was shown to promote translocation of the protein to the nucleus. Collectively our findings suggest that SMTNL1 is a major mediator of both vascular and striated muscle adaptations during pregnancy. In striated muscle, these adaptations involve remodeling of the muscle phenotype towards formation of type IIb fast twitch glycolytic fibers. Our findings also suggest previously unrecognized roles for ER/PR in mediating adaptations in both vascular and striated muscles.

1063/B271

The Pro-Apoptotic Protein Par-4 Regulates Myosin Phosphatase Activity.

S. Vetterkind[1], E. Lee[2], E. J. Sundberg[2], T. C. Tao[2], U. Preuss[3], K. G. Morgan[1]; [1]Sargent College, Boston University, Boston, MA, [2]Boston Biomedical Research Institute, Watertown, MA, [3]University of Bonn, Bonn, Germany

Prostate apoptosis response-4 (Par-4) is a protein that has primarily been connected with apoptotic processes. Recently, we have shown that Par-4 also plays a role in smooth muscle contractility. Here, we have tested the hypothesis that Par-4 regulates the phosphorylation state of myosin regulatory light chain (LC20) by modulating the activity of myosin phosphatase. In the smooth muscle-derived cell line A7r5, Par-4 colocalizes with the targeting (MYPT1) and catalytic (PP1cδ) subunits of myosin phosphatase on actin filaments. Proximity ligation assays demonstrate a close proximity of Par-4 and MYPT1 in vivo. Moreover, endogenous MYPT1 and PP1cδ co-immunoprecipitate with endogenous Par-4 from A7r5 lysates. Direct binding of Par-4 and MYPT1 is shown by surface plasmon resonance, and the leucine zipper of MYPT1 is required for direct binding. The domain of Par-4 that mediates interaction with MYPT1 has been mapped to the leucine zipper motif in co-immunoprecipitation experiments and in proximity ligation assays. LC20 phosphorylation assays using the proximity ligation assay revealed that overexpression of Par-4 and a phosphorylation site mutant of Par-4 (T155A), but not a leucine-zipper defective mutant (L3A), leads to reduced phosphorylation levels of LC20, suggesting activation of myosin phosphatase by Par-4 in a leucine-zipper dependent manner. Moreover, the co-expression of either Par-4 mutant, but not wild type Par-4, with zipper-interacting protein kinase (ZIPK) interferes with ZIPK mediated LC20 phosphorylation. Our results demonstrate that Par-4 interacts with subunits of the myosin phosphatase in vivo, possibly as an accessory protein that supports the catalytic activity of myosin phosphatase by an as yet unknown mechanism. At the same time, Par-4 is required for efficient inhibition of myosin phosphatase by ZIPK. Support: HL31704, HL80003, HL86655, AR41637 from the NIH, AHA postdoctoral fellowship to SV.

1064/B272

Role of Dynamin-2 in Vertebrate Neuromuscular Development.

E. M. Gibbs[1], A. S. Busta[1,2], J. Dowling[2], E. L. Feldman[1]; [1]Neurology, University of Michigan, Ann Arbor, MI, [2]Pediatrics, University of Michigan, Ann Arbor, MI

Dynamin-2 is a ubiquitously expressed protein that plays a key role in vesicle fission during endocytosis. Recently, mutations in dynamin-2 were found to cause two human neuromuscular disorders, a dominant form of centronuclear myopathy and a dominant form of Charcot-Marie-Tooth disease. While dynamin-2 has been extensively studied in intracellular processes, its specific function in nerve and muscle development is unclear. In order to examine this question, we looked at the zebrafish homologue of human dynamin-2. Because of their rapid development and experimental accessibility, zebrafish provide an excellent system for dissecting the role of dynamin during development. In this study, we used morpholino-mediated knockdown of dynamin-2 to determine the effect of dynamin-2 insufficiency on developing zebrafish embryos. We also overexpressed human dynamin-2 constructs with disease-associated mutations, and treated embryos with chemical inhibitors of dynamin. Overall, dynamin-2 knockdown caused global abnormalities, including a shortened body structure and underdeveloped head and eyes. It also resulted in severe edema within the first 48 hours of life, despite the presence of cardiac contractions. Our subsequent analysis focused on skeletal muscle development during embryonic and early larval stages. Dynamin-2 morphants display severe motor impairments, with significant reductions in both spontaneous contractions and touch-evoked movement. Histology of fast and slow muscle revealed highly disorganized myofibers. In all, our studies will lead to a greater understanding of dynamin's role during development, and its role in human neuromuscular disease.

1065/B273

The Effect of Disease Causing Mutations on Sarcomeric Myosin Structure and Function.

T. Armel, L. Leinwand; MCD Biology, University of Colorado, Boulder, CO

Myosin is the molecular motor of muscle and the structural backbone of the thick filament. Many disease-causing mutations have been discovered in the β-myosin heavy chain gene, which is abundantly expressed in heart and skeletal muscle. While the majority of mutations are located in the motor domain, a number (79) of novel mutations in the coiled coil rod region of β-MyHC have been reported. Although the sequences governing activity of the motor domain have been investigated in detail, relatively less is known about the sequence requirements and mechanisms governing thick filament assembly. This study represents the first attempt to characterize the mechanism(s) of pathogenesis of these mutations. Using an assortment of biophysical assays, we investigated the functional effects of seven mutations that give rise to various clinical phenotypes such as hypertrophic and dialated cardiomyopathies, early onset distal myopathy, and myosin storage myopathy. We show there is a broad spectrum of in vivo phenotypes among the seven mutations tested. Circular dichroism experiments suggest an R1500P substitution in the f position of the coiled coil affects the α-helical content of the protein, while a L1793P substitution in the d position shows no effect. Thermal melts and calorimetry suggest these mutations, along with an R1500W substitution alter the thermal stability. Functional assays using 90 degree light scattering and paracrystal formation to follow self-assembly of the proteins show defects in the ability of L1793P and E1886K proteins to associate into the ordered structures formed by WT myosin. The other three mutations show no detectable phenotype in the assays used. Taken together, these data provide evidence for the underlying cause of several cardiac and skeletal myopathies as well as for the importance of these regions of myosin in proper function of the protein.

1066/B274

Characterization of the Non-kinase Activity of Smooth Muscle Myosin Light Chain Kinase.

C. Xie[1], A. Nakamura[1], Y. Zhang[1], Y. Gao[2], R. Ishikawa[1], S. Yoshiyama[1], K. Kohama[1]; [1]Molecular and Cellular Pharmacology, Gunma University Graduate School of Medicine, Maebashi, Japan, [2]Biochemistry and Molecular Biology, Dalian Medical University, Dalian, China

Smooth muscle(sm) myosin light chain kinase (smMLCK) phosphorylates 20kDa myosin light chain (MLC20) by a Ca^{2+}/calmodulin (CaM)-dependent manner and initiates smooth muscle contraction. In addition to the kinase activity, smMLCK also has non-kinase activities that derive from the actin-binding activity at N-terminal region and myosin-binding activity at C-terminal region[1)]. We expressed smMLCK which was furnished with both the kinase and non-kinase activities in E.coli in a large amount[2)]. For the specific analysis of the non-kinase activity, we generated the kinase-dead smMLCK by the site-directed mutagenesis of the ATP-binding sites, producing smMLCK devoid of the kinase activity. Effects of this kinase-dead smMLCK on unphosphorylated myosin were examined by measuring the ATPase activity of sm myosin. We showed that the kinase-dead smMLCK activated Mg^{2+}-ATPase activity of the myosin whether or not actin-filaments were present. We interpret that the non-kinase activity of smMLCK can activate sm myosin without regulatory MLC20 phosphorylation and speculated that myosin-binding activity may play a major role. The effect of Ca^{2+}/ CaM on the non-kinase activity will be examined. (References) 1. A. Nakamura, C. Xie, Y. Zhang, Y. Gao, HH. Wang, LH. Ye, H. Kishi, T. Okagaki, S. Yoshiyama, K. Hayakawa, R. Ishikawa, K. Kohama. (2008) Biochemical and Biophysical Research Communications, 369, 135-143 2. A. Nakamura, K. Kawano, Y. Gao, Y. Hanyuda, A. Takahashi, R. Ishikawa, K. Kohama.(2005) Abstract of 45th American Society for Cell Biology Annul Meeting. 916, B139

1067/B275

Coordinate Regulation of the Smooth Muscle Actin (SMA) Gene by the Barx2 Homeobox Protein and Muscle Regulatory Factors.

H. Makarenkova[1,2], K. N. Gonzalez[3], W. B. Kiosses[4], R. Meech[5]; [1]The Neurosciences Institute, San Diego, CA, [2]Department of Neurobiology, The Scripps Research Institute, La Jolla, CA, [3]Molecular Diagnostic Services, Inc, San Diego, CA, [4]Core Microscopy Facility, The Scripps Research Institute, La Jolla, CA, [5]Department of Clinical Pharmacology, Flinders University, Bedford Park, SA, Australia

Remodeling of the actin cytoskeleton is a critical early step in skeletal muscle differentiation. Smooth muscle actin (SMA) is one of the earliest markers of myoblast differentiation and is important for the migration and cell shape changes that precede fusion. We have found that satellite cell-derived primary myoblasts from mice lacking the Barx2 homeobox gene show altered patterns of actin remodeling, less cell spreading and delayed differentiation. Consistent with the role of SMA in these processes, Barx2 -/- myoblasts also show reduced expression of SMA mRNA and protein. The proximal SMA promoter contains binding sites for muscle regulatory factors (MRFs) and serum response factor (SRF), as well as a conserved homeodomain binding site (HBS). We found that Barx2 binds to the HBS element and upregulates SMA promoter activity in primary cells and myogenic cell lines. We also show that Barx2, MyoD and SRF simultaneously occupy the SMA promoter in cells, and that Barx2 interacts with MyoD. Together these data indicate that Barx2 cooperates with other muscle regulatory factors to regulate the early cytoskeletal remodeling events that underlie efficient myoblast differentiation.

Microtubule-associated Proteins II (1068 – 1083)

1068/B276

State of the Art Differential Proteomics to Identify and Characterize Unknown Microtubule Associated Proteins Regulating Spindle Assembly or Disassembly.

F. Baerenz, S. Merker, T. Ruppert, O. J. Gruss; ZMBH, University of Heidelberg, Heidelberg, Germany

During mitosis, the two newly forming daughter cells must receive one copy of each chromosome. To accomplish this task, the mitotic spindle pulls sister chromatids toward opposite poles of the dividing cell. This microtubule-based structure comprises dynamic polymers made of tubulin heterodimers, associated with a large variety of microtubule-associated proteins (MAPs). A large number of proteins associate with the mitotic spindle and regulate its dynamic formation and function. Stabilizing and destabilizing proteins control the high turnover rate of mitotic microtubules. Maturation (meiosis) of Xenopus laevis oocytes is a particular model system for M-phase entry. After an extensive growth phase, Xenopus oocytes first transiently arrest before meiosis. Upon entering meiosis this arrest is released and a concomitant protein-pattern of MAPs is mediated by specific translation activity of essential proteins needed for meiotic cell division. Before finishing meiotic cell cycle the mature eggs arrest in meiosis two. We applied state of the art differential mass-spectrometry using the isotope coded protein label (ICPL mass-spectrometry) to characterize the Xenopus laevis microtubule-associated proteome comparing and characterizing the MAP pattern of Xenopus stage VI oocytes with the mature eggs. In four independent experiments we identified over 500 microtubule-bound proteins. 20% are down regulated during oocyte maturation whereas 30% are up regulated. Categorizing these candidates evidently shows that not only well characterized MAPs like TPX2, XMAP215, Eg5 and MCAK are up regulated, but also RNA and DNA-binding proteins as well as translation regulating proteins. Taken together these results suggest that protein translation occurs on the spindle during meiosis in the Xenopus oocyte in a maturation dependent manner. The eukaryotic translation inition factor 3 subunit 10 (eIF3s10 / p150) as a potential candidate gives an example as a new role for eIF3S10, possibly by acting as an adapter molecule between the translation initiation apparatus and the cytoskeleton structure of the cell.

1069/B277

Mitochondrial Dysfunction Induces the Accumulation of Microtubule-Associated Protein and Actin Cytoskeletal Components in Alzheimer-Like Striated Neurites.

I. T. Whiteman[1, 2], K. Cullen[2], S. T. Antao[3], P. K. Witting[3], J. Bamburg[4], C. Goldsbury[1]; [1]Brain and Mind Research Institute, University of Sydney, Sydney, NSW, Australia, [2]Discipline of Anatomy and Histology, Bosch Institute, University of Sydney, Sydney, NSW, Australia, [3]ANZAC Research Institute, University of Sydney, Sydney, NSW, Australia, [4]Department of Biochemistry and Molecular Biology, Colarado State University, Fort Collins, CO

Hyperphosphorylation and accumulation of the microtubule-associated protein (MAP) tau into striated neuropil threads is a key neuropathological characteristic of Alzheimer's disease (AD). Although the mechanisms involved in formation of these structures remain poorly understood, the extent of tau pathology can be directly correlated to cognitive decline and disease progression. Since AD is associated with mitochondrial dysfunction and reduced energy utilization, we asked whether these events could trigger changes in MAP phosphorylation and localization in primary neuronal cell cultures. We demonstrate that mitochondrial dysfunction causes the rapid accumulation of phosphorylated MAPs into striated rod-like structures along neurites. These rods resemble neuropil threads observed in postmortem AD brains labeled with the same tau antibodies. We found the inclusions to also contain actin, actin-depolymerizing-factor and cofilin. Taken together, these observations indicate that Alzheimer-like accumulation of phosphorylated MAP is linked to the actin cytoskeleton. Further, classic hyperphosphorylated-tau- and actin-neuropathologies could be associated with mitochondrial dysfunction as an early triggering event influencing microtubule and actin cytoskeletal dynamics in AD neurodegeneration.

1070/B278

Microtentacles are Stimulated by Expression of the Microtubule-Associated Protein Tau in Detached Breast Tumor Cell Lines.

M. A. Matrone, R. Whipple, E. Cho, E. Balzer, S. S. Martin; University of Maryland School of Medicine, Baltimore, MD

The sensitivity of breast cancer to chemotherapy is a primary concern for clinicians and patients. Though treatment regimes greatly increase the rates of disease-free survival, there remains great variability in cancer sensitivities. To combat this, numerous studies have been undertaken to discover predictive molecular markers of breast cancer chemotherapy response. One marker to emerge from such a program is the microtubule-associated protein (MAP), tau. Tau is a classically neuronal MAP that binds to, stabilizes, and promotes polymerization of microtubules. In expression profiles of paclitaxel sensitive and resistant breast cancers, tau was differentially expressed. Tau mRNA expression directly correlated with paclitaxel sensitivity and inversely correlated with patient outcome. The mechanism for this response has been attributed to competition of tau and paclitaxel for binding to the microtubule polymer. To our knowledge, little is known of how tau expression impacts cancer invasiveness and metastasis. Our research focuses on unique tubulin microtentacles that form in detached breast tumor cells and that are implicated in the binding of circulating tumor cells to blood vessel walls during extravasation. We have demonstrated that microtentacles, enriched in detyrosinated tubulin and vimentin, facilitate the reattachment of suspended cells to other cells and to an extracellular matrix. This trait is enhanced in cells with a compromised actin cortex. We examined breast tumor cell lines of varying metastatic potential and found that those endogenously expressing high levels of tau showed extensive microtentacle formation upon suspension and actin disruption. To investigate the role of tau in microtentacle formation, tau was expressed in a panel of cell lines with increasing tau expression. In these cells, exogenous tau expression significantly increased microtentacle formation upon suspension and resembled cell lines with endogenously high tau expression. Exogenous tau expression also altered microtentacle morphology upon suspension, inducing longer, thicker, and more rigid microtentacles. This indicates a potential mechanism by which tau expression can influence metastasis of breast tumor cells.

1071/B279

Synergistic Action of CDK5 and GSK3β Hyperphosphorylate Not Only SP/TP Motif in the Proline-Rich Flanking Region but Also Serine in the KXGS Motif in the Microtubule-Binding Domain of Tau Protein.

M. Hashiguchi[1], T. Hashiguchi[2]; [1]Physiology, Tokyo Medical Univeristy, Shinjuku, Japan, [2]Apis Laboratory, Kitamoto, Japan

The isoform complexity of the microtubule-associating protein, tau, arises from its posttranslational modification. Different phospho-isoforms are expressed in neurons during fetal and adult development, suggesting that phosphorylation of tau plays an essential role in age-related remodeling of neurons. 2D phospho-peptide mapping revealed that simultaneous application by p35-CDK5 and GSK3β evoked stimulation of enhanced bis-phosphorylation of tau at Ser202/Thr205 or Ser199/Thr205, which are major epitopes for AT8. A sequential phosphorylation by GSK3β after the conditioning phosphorylation by p35-CDK5 evoked a striking enhancement of bis-phosphorylation at Ser202/Thr205 or Ser199/Thr205, while the reverse application failed to enhance any phosphorylatable sites. An occlusion assay using 2D mapping revealed that p35-CDK5 primes tau phosphorylation for subsequent GSK3β activity. Both p35-CDK5 and p25-CDK5 can readily incorporate just one phosphate into Ser199 or Ser202 of a tau molecule, but p35-CDK5 poorly introduces the second phosphate into an adjacent Ser/Thr residue adjacent to a phosphorylated one. In contrast, GSK3β conforms to the phospho-directed mechanism so that CDK5 can prime naive tau for the subsequent GSK3β action. Either p35-CDK5 or GSK3β alone weakly phosphorylates the microtubule-binding domain of tau at its Ser324 residue. However, simultaneous application of p35-CDK5 and GSK3β resulted in a dramatic enhancement of Ser324 phosphorylation. Synergistic action induced by sequential action of CDK5 and then GSK3beta hyperphosphorylates tau as a functional unit.

1072/B280

Cooperative regulation of microtubule and focal adhesion dynamics by APC tumor suppressor and DDEFs.

C. Matsui[1], S. Kaieda[2,1], T. Ikegami[2], Y. Mimori-Kiyosue[1]; [1]Cytoskeleton & Cell Motility Research Group, KAN Research Institute, Kobe, Japan, [2]Laboratory of Structural Proteomics, Institute for Protein Research, Osaka University, Suita, Osaka, Japan

The APC tumor suppressor protein is one of +TIPs (microtubule plus-end-tracking proteins) affecting the behavior of microtubule plus ends. We have reported that DDEF1 and DDEF2, being ArfGAPs towards Arf1 and Arf6, interact with the SAMP region through their SH3 domains. DDEFs are known to associate with focal adhesion proteins, such as paxillin and FAK, and regulate focal adhesion dynamics. The NMR (nuclear magnetic resonance) chemical shift perturbation study demonstrated that the SAMP motif interacts in almost the same region of DDEF-SH3 as FAK, suggesting that the binding of APC to DDEFs could compete with FAK. Since microtubules have been thought to promote focal adhesion turnover, it is interesting to investigate the involvement of APC-DDEF complex in the focal adhesion dynamics. In *Xenopus* A6 epithelial cells, overexpression of GFP-tagged full length APC, accumulating at the microtubule ends extending to the cell edges, facilitated the turnover of focal adhesions relying on the SAMP repeat region, and this effect was suppressed by overexpression of DDEFs. Also RNAi experiments in human MCF10A epithelial cells showed that DDEFs suppress the invasion of microtubules into the lamellipodia, while APC promotes microtubule growth. A model of the cooperative activity between APC and DDEF will be discussed.

1073/B281

Ca2+ Signaling Regulates the Subcellular Localization of Adenomatous Polyposis Coli Tumor Suppressor Protein.

T. Togo; Department of Anatomy, St. Marianna University School of Medicine, Kawasaki, Japan

The microtubule (MT) plus end tracking proteins such as adenomatous polyposis coli (APC) and CLIP-170 associated proteins (CLASPs) are involved in the regulation of MT plus-end dynamics and stabilization. It has previously been reported that disruption of plasma membrane and increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) stimulates rearrangement of MTs (Togo, 2006, J. Cell Sci. 119:2780-2786). To understand how increase in $[Ca^{2+}]_i$ regulates dynamics of MTs, behaviors of APC and CLASPs were observed under the various conditions using *Xenopus* A6 epithelial cell transfectants expressing GFP-tagged APC and CLASP1/2. Disruption of plasma membrane and increase in $[Ca^{2+}]_i$ stimulates dissociation of APC from MTs through CaMKII-, tyrosine kinase-, and GSK-3β-dependent pathway, whereas CLASPs did not respond to increase in $[Ca^{2+}]_i$. When highly polarized quiescent monolayers of parental A6 cells were scratched, both elongation of MTs and directional cell migration toward the wound were suppressed by inhibiting CaMKII, tyrosine kinase, and GSK-3β. Since the plasma membrane of the cells lining scratch site was temporally disrupted, the present study suggests Ca^{2+} signaling facilitates cell migration by stimulating rapid redistribution of APC after scratching monolayers.

1074/B282

Regulation of Adenomatous Polyposis Coli Protein and Microtubule Organization by Activation of the MAPK Pathway during NGF-Induced Neurite Extension in PC12 Cells.

H. Y. Caro-Gonzalez[1], L. N. Nejsum[2], W. J. Nelson[2,1], A. I. Barth[2,1]; [1]Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA, [2]Department of Biology, Stanford University, Stanford, CA

Adenomatous polyposis coli (APC) is a multifunctional protein that can bind directly to microtubules (MTs), but is clustered at sites of MT-cortex interactions during membrane extension in several cell types. Mechanisms regulating APC localization and function with MTs are poorly understood. Previous studies indicated that local inhibition of GSK3β at the leading edge of membrane extensions in astrocytes and neurons is necessary for APC clustering and membrane extension, probably by inhibiting its phosphorylation which increases MT bundling by APC. We investigated mechanisms of APC regulation during neurite extension in PC12 cells induced by nerve growth factor (NGF), which involves the activation of protein kinase signaling cascades and MT reorganization. We show that APC is rapidly phosphorylated upon NGF stimulation of PC12 cells, and that this correlates with the formation of APC cortical clusters at the tips of initial membrane extensions. Pharmacological inhibition of GSK3β did not induce APC phosphorylation or clustering at extension tips and also had no significant effect on APC phosphorylation in response to NGF. APC cluster formation and phosphorylation strongly increased when cells were stimulated with NGF and forskolin, which increases cAMP levels in cells, and these effects were blocked by pharmacological inhibition of the mitogen activated protein kinase (MAPK) pathway. Several putative MAPK phosphorylation sites are located in the C-terminus of APC, close to sites important for APC interactions with MT. Significantly, S →A substitution of one of those sites dramatically decreased APC cortical

clustering and promoted APC localization along MTs. These data indicate that NGF-induced APC phosphorylation by the MAPK pathway causes APC reorganization to form cortical clusters at the ends of MTs needed for neurite extension.

1075/B283

Characterization of C. elegans CLASP2.

A. Otsuka[1], P. Gong[1], C. MacDonald[1], J. Ahn[2], G. Machekanyanga[1], S. Burge[1], M. Lee[2]; [1]Department of Biological Sciences, Illinois State University, Normal, IL, [2]Department of Biology, Baylor University, Waco, TX

CLASP2 is found at the plus ends of microtubules in the leading edges of growth cones and migrating cells, as well as associated with the kinetochore during cell division. We have characterized CLASP2 by confocal microscopy using either CLASP2 antibodies (kind gift of A. Desai and K. Oegema) or via transgenic CLASP2-GFP fusions. There were similarities and differences in the patterns of localization between endogenous and transgenic CLASP2. In the cells of transgenic embryos, there were bundled peripheral loops of CLASP2-GFP-labeled microtubules that are not seen in non-transgenic animals; however, CLASP2 over expression is known to cause microtubule bundling in other systems. The pattern of CLASP2 antibody (Ab) staining changed from cytoplasmic in interphase cells to perichromosomal in dividing cells. The centrosomes also stained in many cells. While phalloidin-FITC did not strongly stain the cell periphery, CLASP2 staining was observed at the cell cortex. In the hermaphrodite gonad, CLASP2 Ab stained the rachis and ring-like structures around the developing germinal vesicles. In newly fertilized eggs, CLASP2 labeled the meiotic spindle and wisps at the cell surface. In larval stages, there was strong CLASP2 Ab staining at the apical junctions of intestinal cells, a few somatic cells, and at the nerve ring. For transgenic animals, CLASP2-GFP expressed from the CLASP2 promoter was found in several cell types, including neurons, muscle, and intestinal cells. CLASP2-GFP was strongly expressed in gland cells, such as pharyngeal g1 and g2, posterior glands, and the excretory canal cell.

1076/B284

GSK3β Phosphorylation Modulates CLASP-Microtubule Association and Lamella Microtubule Attachment.

P. Kumar[1], K. Lyle[1], S. Gierke[1], A. Matov[2], G. Danuser[2], T. Wittmann[1]; [1]Cell and Tissue Biology, University of California San Francisco, San Francisco, CA, [2]Cell Biology, The Scripps Research Institute, La Jolla, CA

Polarity of the microtubule cytoskeleton is essential for many cell functions, and this polarity is determined by microtubule interactions with the cortical cytoskeleton. CLASPs are good candidates to mediate such cortical microtubule interactions, because CLASPs bind cortex-associated proteins such as LL5β, and stabilize microtubules in the cell periphery. In addition, CLASPs track microtubule plus ends in the cell body, but bind along microtubules in the lamella of migrating epithelial cells. This unique spatiotemporal regulation is mediated by two separate microtubule-binding domains. Because CLASP-microtubule association is negatively regulated by GSK3β phosphorylation, we hypothesized that GSK3β may be a master regulator of lamella microtubule dynamics. To test this, we quantified microtubule dynamics by manual and computer-vision tracking of microtubule ends, and found that expression of constitutively active GSK3β(S9A) specifically decreased the stability of lamella microtubules. To determine whether CLASPs are responsible for this GSK3β-dependent regulation of microtubule dynamics, we analyzed CLASP2 phosphorylation by GSK3β in cells and in vitro. We identified eight serine residues in the plus-end-tracking domain that can be directly phosphorylated by GSK3β. Phosphorylation of these serines directly inhibited binding of the CLASP2 plus-end-tracking domain to the negatively charged tubulin COOH-terminus by disrupting electrostatic interactions. However, we also found that the phosphorylation state of a subset of these serine residues that are phosphorylated under physiological levels of GSK3β activity determines whether full-length CLASP2 tracks plus ends or associates along microtubules, indicating a long-range effect of CLASP2 phosphorylation on the second microtubule-binding domain. Finally, GSK3β-induced lamella microtubule destabilization was rescued by expression of CLASP2 with mutated phosphorylation sites, indicating that CLASPs play a major role in lamella microtubule stability, which may be regulated by local GSK3β inactivation.

1077/B285

Gene Expression Profiles of HCT116p53-/- Cells Treated with Paclitaxel or Stathmin-siRNA.

D. Ringhoff[1,2], B. Carney[2], A. Lefkowitz[3], E. Smith[4], J. Marzillier[2], P. Wu[3], L. Cassimeris[2]; [1]Chemistry, Lehigh University, Bethlehem, PA, [2]Biological Sciences, Lehigh University, Bethlehem, PA, [3]Mathematics, Lehigh University, Bethlehem, PA, [4]Bio-Engineering, Lehigh University, Bethlehem, PA

Among the most difficult cancers to treat are those having mutated or non-functional p53, with concurrent overexpression of stathmin (stmn1), a microtubule (MT) destabilizer (e.g. Yuan et al. 2006. J. Pathol. 209: 549-8). Alli et al. (2007. Oncogene. 26:1003-12) demonstrated that this type of cancer cell undergoes apoptosis in response to stmn1 knock down by siRNA. The mechanism by which stmn1 siRNA promotes apoptosis in cancerous cell lines is not understood, while efficient delivery of targeted siRNA therapies remains an elusive challenge. In this work, we aim to understand the apoptotic processes activated by stmn1 siRNA in a p53-null human colon cancer cell line, HCT116p$^{53-/-}$, and specifically what is unique about stmn1 siRNA induced apoptosis compared to that induced by paclitaxel treatment, a MT stabilizing drug. We find that both stmn1 depletion and paclitaxel addition slowed cell growth and caused ~25% cell death beginning ~48 h after initial treatment. Microarray analyses of gene expression profiles were then examined to provide an unbiased screen for genes up or down regulated by either treatment. Time courses (~55-72 h) were analyzed for annotated genes showing expression fold changes of ≥2.0. In stmn1 siRNA treated cells, we found 215 down-regulated and 157 up-regulated genes. Paclitaxel treatment resulted in 162 down-regulated and 642 up-regulated genes. Of the differentially regulated genes from each treatment, the number of shared genes included 27 of 350 (8%) down-regulated genes and 89 of 710 (13%) up-regulated genes. These data indicate that most expression changes were unique to each treatment. Comparing those genes whose expression is oppositely changed between the two treatments also demonstrated a low level of overlap (95 of 1081 genes, or ~9%) between treatments. To date our results indicate that stmn1 siRNA and paclitaxel induce apoptosis through unique upstream signals, although each treatment acts to stabilize MTs. Continued analysis and microarray verification are ongoing to confirm our conclusions. Understanding how stmn1 siRNA induces apoptosis could lead to identification of novel chemotherapy targets. Partially funded by an HHMI grant to Lehigh and NIH (LC).

1078/B286

Stathmin/Oncoprotein 18 Is Required for Cell Survival In Cells Lacking P53.

B. Carney, L. Cassimeris; Biological Sciences, Lehigh University, Bethlehem, PA

Cancer cells often over-express stathmin/oncoprotein 18, a microtubule regulatory protein. Several groups have demonstrated previously that depleting stathmin from some, but not all, cancer cell lines leads to apoptosis. Those cancer cell lines which die after stathmin depletion are correlated with loss of functional p53 (Alli et al. Oncogene, 2006.), but whether p53 loss is required for the stathmin-depletion induced cell death has not been determined. We asked whether stathmin depletion induces apoptosis only in cells without p53 by comparing the consequences of stathmin depletion from matched cell lines differing only in p53 status. First, we examined stathmin-depleted HCT116 colon cancer lines +/+ or -/- for p53. In HCT116 p53-/- cells, ~75% of stathmin is depleted 1-2 days after siRNA transfection; cells die 2 - 5 days after siRNA transfection. In contrast, stathmin in HCT116 p53+/+ cells is more difficult to deplete; we find that stathmin level is reduced > 50% by 3 - 5 days after siRNA transfection. In these WTp53 cells, stathmin depleted cells continue to grow at normal rates for at least 7 days after siRNA transfection. As a second experimental system, we are also comparing the effects of stathmin depletion from HeLa cells, which normally lack p53 (due to expression of the HPV E6 protein), to HeLa cells expressing p53 (by siRNA-mediated depletion of E6). We find that HeLa cells (without detectable p53) begin to die 2 to 3 days after stathmin siRNA transfection. Restoring p53 level by E6 depletion rescues HeLa cells from stathmin-depletion induced death; these cells survive for at least 7 days although their growth is slowed compared to cells expressing stathmin. For both HCT116-/-p53 and HeLa cells, the cell death occurring after stathmin depletion appears to be via apoptosis based on preliminary evidence of PARP cleavage and annexin V translocation to the outer leaflet of the plasma membrane. Our results demonstrate that stathmin is required for cell survival only in cells lacking p53, suggesting that stathmin depletion could be used therapeutically to induce apoptosis in tumors without a functional p53. Thanks to B. Vogelstein for HCT116 lines and NIH for support.

1079/B287

3D Electron Microscopy of Interphase Microtubule Assemblies in Ase1p and Klp2p Deletion Strains in Fission Yeast.

H. Roque[1], D. Brunner[1], D. Mastronarde[2], C. Antony[1]; [1]Cell Biology and Biophysics, EMBL, Heidelberg, Germany, [2]The Boulder Laboratory of 3D EM of cells, and Developmental Biology, University of Colorado, Boulder, CO

Microtubule (MT) assemblies are essential for basic cellular processes such as organelle positioning and cell shape control. We made use of Ase1p and Klp2p deletion mutants of the fission yeast Schizosaccharomyces pombe to study the organization of interphase microtubule assemblies (IMAs) in high ultrastructural detail, using electron tomography (ET). High resolution 3D volumes of Ase1p and/or Klp2p deletion strains were reconstructed. We show that cells lacking Ase1p maintain MT overlap regions but with altered inter-MT spacing. IMAs in klp2Δ cells are mostly composed of only two MTs instead of 2 to 9 as in wild type. In ase1Δ klp2Δ cells, we also observe MT overlap regions with altered inter-MT spacing. In addition, MTs appear to have lost their connection to the spindle pole body (SPB). Finally, we show that impaired IMAs affect the mitochondria network structure in these deletion strains.

1080/B288

Growth-arrest-specific Protein 2 Stops Cell Division by Disrupting Microtubules Dynamics.

T. Zhang[1], C. Mandato[1,2]; [1]Department of Biology, McGill University, Montreal, QC, Canada, [2]Department of Anatomy and Cell Biology, McGill University, Montreal, QC, Canada

The growth-arrest-specific 2 (*gas2*) gene was initially identified from high levels of expression in murine fibroblasts under growth arrest conditions. It was also found that *gas2* is down-regulated upon serum stimulation, which leads to re-entry into the cell cycle. It has been shown that the *gas2* gene product-Gas2 protein, may interact with both F-actin and microtubules. In this study, full-length Gas2 protein was microinjected into *Xenopus laevis* embryos, and found to prevent subsequent cell divisions. Cryo-confocal microscopy analysis also suggested that Gas2 overlaps with microtubules. Electron microscopy further revealed that the purified full-length Gas2 can bundle microtubules. Furthermore, a cytokinesis analog contractile array assay showed that Gas2 co-localizes with double F-actin rings during the cell wound healing, which is a typical phenotype seen when the *Xenopus laevis* oocytes are treated with microtubule stabilization drug Taxol. Structure function analysis demonstrated that Gas2 can stabilize microtubules via its c-terminal Gas2 domain. Flow cytometry has shown that Gas2 DNA transfected HeLa cells have a significant increase in multiple nuclei cells in the culture, which suggests that over-expression of Gas2 can result in cytokinesis failure, and the cell cytokinesis failure is caused by the effect of Gas2 on microtubule dynamics.

1081/B289

Structural Insights into the Mechanism of Microtubule Severing.

A. Roll-Mecak, R. Vale; Cellular and Molecular Pharmacology, University of California San Francsico, San Francisco, CA

Microtubules, 25 nm cylindrical polymers that are nearly inflexible on the micron length scale, must dynamically reorganize during processes such as cell migration, neurite outgrowth, and spindle formation. Spastin, the most common locus of mutations in hereditary spastic paraplegias (HSP), and katanin define a subfamily of AAA ATPases that sever microtubules and are important in many settings in cell biology: chromosome segregation, cilia biogenesis and axonal outgrowth and maintenance. Microtubule severing is an energetically demanding molecular task, as both longitudinal and lateral contacts in the microtubule lattice need to be broken. This is extremely puzzling since the microtubule has a lateral rigidity comparable to that of Plexiglas. The mechanism employed by microtubule severing enzymes to internally destabilize the microtubule lattice has remained elusive. We have used X-ray crystallography to solve the atomic structure of the spastin monomer and solution light and X-ray scattering coupled with atomic docking to generate a model for the active spastin hexamer. This model reveals that spastin forms a ring with a prominent central pore and six radiating arms that might dock onto the microtubule. An N-terminal helix, unique to spastin and katanin, surrounds the entrance to the pore and may be allosterically linked to the ATP binding site. Three highly conserved loops line the lumen of the central channel, and disease mutations as well as structure guided mutational analyses reveal that they play an important role in microtubule severing. We suggest a model in which these pore loops act in concert to "tug" on the C-terminal region of tubulin (known to be required for spastin- and katanin-mediated severing), generating a mechanical force that destabilizes tubulin-tubulin interactions within the microtubule lattice, leading to catastrophic breakdown of the microtubule. Roll-Mecak, A.

and Vale, R.D. Structural basis for microtubule severing by the hereditary spastic paraplegia protein spastin. 2007. Nature, 451(7176):363. Roll-Mecak, A. and Vale, R. D. The Drosophila Homologue of the Hereditary Spastic Paraplegia Protein, Spastin, Severs and Disassembles Microtubules. 2005. Curr. Biol. 5(7), 650.

1082/B290

Analysis of Microtubule Severing by the C. elegans Spastin Homologue SPAS-1, a Member of AAA Proteins.

Y. Matsushita-Ishiodori, M. Esaki, K. Yamanaka, T. Ogura; Division of Molecular Cell Biology, Insititute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan

Mutations of human spastin, an AAA (ATPases associated with diverse cellular activity) family protein, cause an autosomal dominant form of hereditary spastic paraplegia, which is characterized by weakness, spasticity and loss of the vibratory sense in the lower limbs. Recently, it has been reported that spastin interacts dynamically with microtubules (MTs) and displays microtubule-severing activity. However, the detailed function and molecular mechanism of spastin remain unknown. A plausible *Caenorhabditis elegans* homologue of spastin (SPAS-1) has been identified by homology search and phylogenetic analysis. To understand the function of the spastin homologue and the molecular mechanism of microtubule-severing, I characterized the deletion mutant of spas-1, which has a 917 bp deletion in the *spas-1* gene, and carried out *in vitro* analysis of purified SPAS-1. I previously reported that in the deletion mutant, MTs were concentrated at the centrosomal region in early embryos, suggesting that *C. elegans* SPAS-1 may be involved in MT dynamics as spastin from human, mouse and fly is so. Furthermore, *in vitro* experiments indicated that α/β-tubulin heterodimer interacts with SPAS-1 directly and MTs stimulate the ATPase activity of wild-type SPAS-1. On the other hand, the AAA domain contains a highly conserved aromatic residue that lies in the central pore region of the hexameric ring. It was reported that the pore in human spastin recognizes the C-terminal region of α-tubulin, which has an acidic tail. So the surface basic residues of SPAS-1 were replaced by alanine. When the central pore mutants were expressed, MT network didn't disappear. However, in the case of non-pore mutants MT network disappeared. These data suggest that the central pore residues are important for the MT disassembly. I also observed the oligomer formation of SPAS-1 in a concentration-dependent manner. I will discuss the function and molecular mechanism of SPAS-1 based on these *in vivo* and *in vitro* results.

1083/B291

Functional Relationship between MCAK and the End-Binding Proteins EB1, EB2, and EB3.

S. Domnitz, L. Wordeman; Physiology & Biophysics, University of Washington, Seattle, WA

There are numerous endogenous cellular proteins that can alter the kinetics of tubulin addition and loss at the ends of microtubules including mitotic centromere-associated kinesin (MCAK) and the End-Binding proteins EB1, EB2, and EB3. MCAK destabilizes microtubules by removing tubulin dimers from microtubule ends. EB1 associates with polymerizing microtubule tips and may interact or compete with microtubule regulators such as MCAK. Less is known about EB2 and EB3, but they too may interact or compete with MCAK and/or each other. We report here that over-expression of Enhanced Green Fluorescent Protein (EGFP)-EB1 or EGFP-EB3 increased MCAK presence on the microtubule lattice and decreased EB2 presence on microtubules as determined by immunofluorescence staining in the primary human fibroblast cell line HEPM. EGFP-EB3 over-expression also reduced the levels of EB1 protein on microtubule tips. These results provide evidence for an interaction between the EBs and MCAK and for microtubule binding competition among the EB proteins.

Actin-associated Proteins II (1084 – 1110)

1084/B292

Formin Specialization in Fission Yeast: Biochemical Study of Mating Formin Fus1p Reveals Unique Actin Assembly Properties That May be Required for Chemotaxis During Mating.

B. J. Scott[1], D. R. Kovar[1,2]; [1]Molecular Genetics and Cell Biology, The Unversity of Chicago, Chicago, IL, [2]Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL

Formins drive actin filament assembly for diverse cellular processes including cell division, polarity, and chemotaxis. Fission yeast, as well as most eukaryotes, contain multiple formin isoforms that are specialized for different tasks. To investigate the mechanism of formin specialization in fission yeast, we directly compared the actin assembly properties of the formin required for chemotaxis during mating (Fus1p) to the formin required for building a contractile ring during cytokinesis (Cdc12p). Like Cdc12p and most other formins, Fus1p nucleates actin filament assembly and remains processively associated with the elongating barbed end while facilitating the addition of profilin-actin faster than actin alone. However, specific properties differ between Fus1p and Cdc12p. Although both fission yeast formins are surprisingly efficient nucleators, the resulting filaments elongate at different rates. In the absence of profilin, Fus1p- and Cdc12p-associated actin filaments elongate at ~0.5-1 and ~0.1-0.5 subunits/s, respectively. Interestingly, in the presence of profilin-actin, Fus1p-associated filaments do not elongate nearly as fast (~4-5 subunits/s) as Cdc12p-associated filaments (~10-12 subunits/s), possibly due to Fus1p containing fewer proline-rich tracks in its FH1 domain. Therefore, both fission yeast formins assemble actin filaments by a similar general mechanism, but our work suggests that specialized formins with carefully tuned actin assembly properties are required for building the different actin structures used during mating and cell division in fission yeast.

1085/B293

Mammalian Diaphanous-Related Formin Dia1 Controls the Organization of E-Cadherin-Mediated Cell-Cell Junctions.

L. Carramusa[1,2], C. Ballestrem[3], Y. Zilberman[2], A. Bershadsky[2]; [1]New Materials and Biosystems, Max Plank Institute for Metal Research, Stuttgart, Germany, [2]Molecular Cell Biology, The Weizmann Institute of Science, Rehovot, Israel, [3]Faculty of Life Science, Wellcome Trust Centre for Cell-Matrix Research, University of Manchester, Manchester, United Kingdom

The diaphanous-related formin Dia1 is a target of RhoA and a potent activator of actin nucleation and elongation. Here, we demonstrate that short hairpin (sh) RNA-mediated downregulation of Dia1 in MCF7 epithelial cells disrupts adherens junctions, as manifested by significantly

decreased localization of E-cadherin and associated proteins to cell-cell contacts. Expression of mouse Dia1, which is insensitive to the human Dia1-specific shRNA, rescued the junctional integrity. Co-expression of GFP-Dia1 and a constitutively active RhoA mutant (RhoA-V14) resulted in the localization of the exogenous GFP-Dia1 to the cell-cell junctions. This localization was accompanied by a strong increase in the width of the adhesion zone and augmentation of the actin, E-cadherin, and β-catenin content in the junctions. A constitutively active Dia1 mutant lacking the N-terminal portion was unable to localize to cell-cell junctions and did not show any junction strengthening effect. Adherens junction (AJ) enhancement induced by Dia1 and active RhoA did not require microtubules, but depended on myosin II activity. Inhibition of myosin II activity abolished the Dia1-mediated reinforcement of cell-cell junctions and instead induced the formation of numerous actin-rich filopodia at the contact zone. Thus, Dia1 localizes to and controls cadherin-mediated junctions in a RhoA-dependent manner.

1086/B294

In Vitro and In Vivo Characterisation of the Biochemical Properties and Biological Function of Daam Formin Domains.

S. Barko[1], J. Mihaly[2], M. Nyitrai[1]; [1]Department of Biophysics, University of Pecs, Pecs, Hungary, [2]Institute of Genetics, Biological Research Center of the Hungarian Academy of Sciences, Szeged, Hungary

In this study we described the interactions of the FH2 and FH1-FH2 fragments of the Drosophila formin DAAM with actin and profilin by using various biophysical methods. We used pyrene fluorescent measurements to describe the polymerization and depolymerization dynamics of the actin filaments in the presence of various concentrations of DAAM FH2 and DAAM FH1FH2, and the effect of these fragments on the critical concentration of the actin. Our data showed that the DAAM fragments increased the polymerization rate and decrease the depolymerisation rate of actin, while had only little effect on the critical concentration for actin assembly. We also described the interaction between actin-binding protein profilin and the FH1 domain of the DAAM. The interactions were observed with DAAM-FH1FH2, but not with DAAM-FH2. These interactions lead to the further acceleration of the polymerization. The actin-binding affinities of the DAAM fragments were determined by cosedimentation experiments. These data show that the DAAM fragments could bind to the side of the actin filaments with micromolar affinities. We used fluorescence microscopic measurements to describe the cross-linking effect of DAAM on the actin filaments. Our observations showed that DAAM-FH1-FH2 induced actin filament bundling by cross-linking the filaments.

1087/B295

Functional Interactions between POPX2 Phosphatase and mDia Modulate Rhoa Pathways.

C. Koh; School of Biological Sciences, Nanyang Technological University, Singapore, Singapore

Rho GTPases and their downstream effectors regulate changes in the actin cytoskeleton that underlie cell motility and adhesion. They also participate in the regulation of gene transcription with RhoA activating transcription from the serum response element (SRE) by serum response factor (SRF). SRF transcription is also promoted by several proteins regulating the polymerization or stability of actin. We have previously identified a family of PP2C phosphatases, POPXs, which can dephosphorylate Cdc42/Rac activated kinase PAK and down regulate its enzymatic and actin cytoskeletal activity. We now report that POPX interacts with the formin protein mDia. This interaction is enhanced when mDia is activated by RhoA. We are able to isolate endogenous POPX2-mDia1 complex by immunoprecipitation. The binding of POPX2 to mDia1 or mDia1-containing complex greatly decreases the ability of mDia1 or RhoA to activate transcription from the SRE. We propose that the interaction between mDia and POPX2 serves to regulate both the actin cytoskeleton and SRF transcription, and to link Cdc42/Rac with RhoA pathways.

1088/B296

Biochemical Characterization and Functional Analysis of a Plant Type-II Formin, AtFH16.

Y. Zhang, J. Wu, L. Meng, H. Ren; Beijing Normal University, Beijing, China

Formins represent a family of multidomain proteins that have been found in all eukaryotes examined and are required for multiple actin-related processes. A subset of plant formins, refered to as type-II, does not contain transmembrane domain in the N-terminus and its function is poorly understood. In this study, we provide the biochemical characterization and functional analysis of a type-II plant formin AtFH16. The purified recombinant AtFH16 (FH2) domain forms dimers and trimers. Compared with other formins, such as AtFH8, the actin nucleation activity of AtFH16 protein is much weaker. However, AtFH16 protein has strong actin bundling ability. AtFH16 (FH2) domain is sufficient to induce actin filament bundles efficiently and FH1 domain increases the ability. In addition, over-expression of AtFH16 in Arabidopsis stimulates supernumerary actin cable formation in the cytoplasm and results in increase of primary root length, lateral root number and leaf blade size. Detailed analysis reveals that the phenotype is attributable to a change in cell number rather than cell size. Based on the observations, we propose that AtFH16 may play an important role in organizing actin filaments into higher-order structures like actin bundles or cables and facilitate the cell proliferation in plant cells.

1089/B297

FMNL1 Maintains Golgi Structure and Regulates Cell Cycle Progression.

J. M. Colon-Franco[1], T. S. Gomez[2], D. D. Billadeau[2,1]; [1]Department of Biochemistry and Molecular Biology, College of Medicine, Mayo Clinic, Rochester, MN, [2]Division of Oncology Research, College of Medicine, Mayo Clinic, Rochester, MN

Formins are a family of proteins that nucleate actin and have been implicated in a variety of cellular processes including vesicular trafficking, migration, endocytosis, filopodia formation, cytokinesis, cell polarity and microtubule dynamics. The formin-like (FMNL) gene family encodes the Diaphanous-related proteins FMNL1, FMNL2 and FMNL3. So far, the roles of these proteins and their mechanisms of regulation have not been heavily investigated. Here, we studied the cellular localization and functions of FMNL1. Immunofluorescence studies revealed that FMNL1 co-localize with Golgi markers throughout the cell cycle. Short hairpin mediated gene suppression of FMNL1 results in Golgi fragmentation. This phenotype is rescued by re-expression of FMNL1, but not by a FMNL1 mutant lacking the actin polymerization domain. Additionally, entry into mitosis is delayed in FMNL1 suppressed cells. Taken together, our data indicates that FMNL1 participates in cell cycle progression by regulating Golgi structure.

1090/B298

Isoform-Specific Contributions of α-Actinin to the Cellular Mechanobiology of Glioblastoma Multiforme.

S. Sen, S. Kumar; U. C. Berkeley, Berkeley, CA

Glioblastoma Multiforme (GBM) is a high-grade astrocytoma associated with low survival rates due to aggressive invasion of the tumor cells into the brain parenchyma. Expression of the actin binding protein, α-actinin, specifically its nonmuscle isoforms α-actinin-1 and α-actinin-4, has been strongly correlated with the invasive phenotype of GBM in vivo. To probe the cellular basis of this correlation, we have used RNA interference to investigate the contributions of α-actinin-1 and α-actinin-4 to the structure, mechanics, and motility of human astrocytoma cells in culture. Knockdown of either isoform leads to increased expression of vinculin in U-373 MG human astrocytoma cells, with increased vinculin localization at focal adhesions only when α-actinin-1 is suppressed. On mechanically compliant (1-2 kPa) collagen-coated polymer gel matrices, these cells are rounded and have a mean cortical stiffness of ~2.5 kPa as measured by atomic force microscopy. As gel stiffness is increased, cortical stiffness rises and then on gels stiffer than 8 kPa. By contrast, knockdown of either isoform reduces mean cortical stiffness to ~1.5 kPa on soft gels with a plateau stiffness of ~2.5 kPa. Time-lapse imaging of cells reveals ~35% reduction in mean cell speed with either α-actinin-1 or α-actinin-4 knockdown, with ~50% reduction obtained upon dual knockdown. While Y27632 (Rho-associated kinase inhibitor) renders the cells more invasive, ML7 (myosin light chain kinase inhibitor) slows them down. However, α-actinin-4 knockdown suppresses Y27632-induced invasiveness, and suppression of motility with ML7 treatment is even more effective when α-actinin-1 is knocked down. Our results demonstrate that both α-actinin-1 and α-actinin-4 make critical but separable contributions to cytoskeletal organization, rigidity-sensing, and motility of astrocytoma cells. This yields mechanistic insight into the observed correlation between α-actinin expression and glioma invasiveness in vivo.

1091/B299

Functional Characterization of Nuclear Alpha-actinin 4.

M. Kumeta, Y. Hirano, K. Hashida, S. Yoshimura, K. Takeyasu; Graduate School of Biostudies, Kyoto University, Kyoto, Japan

Alpha-actinin is a member of the spectrin superfamily of actin-binding proteins, generally involved in actin cytoskeleton. In addition to its role as a cross-linker of actin filament at focal adhesion, alpha-actinin 4 (ACTN4), one of four alpha-actinin isoforms, localized to the nucleus with unidentified function. We have raised a monoclonal antibody (#92B) which specifically recognized nuclear ACTN4. Immunostaining of HeLa cells revealed that the nuclear ACTN4 transiently localized to the cytoplasm at early G2 phase and rapidly re-localized to the nucleus in late G2 phase, suggesting a strict control of its nuclear localization mechanism in a cell cycle-dependent manner. When the coiled region of the molecule was tagged with GFP and expressed in HeLa cells, the signal was detected in the nucleus while the full-length GFP-ACTN4 localized to the focal adhesion. When the cells were treated with Leptomycin B, an inhibitor for NES dependent nuclear export, significant amount of endogenous ACTN4 accumulated to the nucleus. These results suggest that ACTN4 is a shuttling molecule between nucleoplasm and cytoplasm. Interestingly, the nuclear ACTN4 formed specific foci on chromosome during mitosis. Co-staining of the mitotic chromosome spreads with one of the rRNA transcription factor UBF clearly revealed that the foci corresponded to the nucleolar organizer regions. It is also confirmed by co-precipitation assay that ACTN4 is involved in rRNA transcriptional machinery during mitosis. These novel findings may reveal the dynamic relationships between cytoskeleton and the nucleus/nucleolus.

1092/B300

Alpha-actinin Promotes PI3 Kinase Associated Growth Arrest in Astrocytoma Cells.

Q. Quick[1], O. Skalli[2]; [1]Biological Sciences, Grambling State University, Grambling, LA, [2]Anatomy and Cell Biology, Lousiana State University Health Sciences Center, Shreveport, LA

Objective: In the last thirty-five years primary malignant brain and central nervous system tumors have had an ~25% relative survival rate of five years post-diagnosis, suggesting that the intrinsic resistance of these cancers to current clinically used treatment protocols are marginally effective in curing this disease and preventing tumor recurrence. In the present study we assessed the role of a-actinin, an actin bundling protein abundantly expressed in highly motile cells, as a contributing factor during cellular motility and mitogenesis in astrocytomas. Results: Using immunoblotting procedures and confocal microscopy analysis we demonstrated that a-actinin 4 was expressed in several established glioma cell lines and primary glioma cells cultured from tumor patient explants. Subsequently, we examined the response of astrocytoma cells deficient for a-actinin using an RNA-interference approach. Boyden chamber experiments revealed that down-regulation of a-actinin did not inhibit the motility of astrocytoma cells. However clonogenic survival studies revealed a reduction in proliferative capacity of astrocytoma cells with decreased levels of a-actinin 4, which was accompanied by increased beta-gal staining, an indicator of senescence arrest and decreased levels of the pro-survival protein PI3-kinase, as compared to controls. Conclusion: Taken together these data suggest that the pro-tumorigenic function of a-actinin in astrocytoma cells is associated with mitogenesis and that abrogating this function delays cellular growth and/or promotes growth arrest in these tumors.

1093/B301

The Phosphorylation of Alpha-Actinin Stimulated with Epidermal Growth Factor Is Regulated by the Interaction with Actin.

H. Shao, C. Wu, A. Wells; Pathology, University of Pittsburgh, Pittsburgh, PA

Alpha-Actinin is a ubiquitously expressed protein well known for its role in cross-linking actin filaments, whose regulation has been linked to cell adhesion and motility. The consequences of growth factor-induced phosphorylation of actinin, however, remain unclear. We expressed two isoforms of human alpha-actinin (1 and 4) in murine fibroblasts which express human epidermal growth factor receptor (EGFR) and found that both alpha-actinin 1 (ACTN 1) and alpha-actinin 4 (ACTN 4) were phosphorylated on tyrosine residues after stimulation with epidermal growth factor (EGF). This occurred through a p38-MAPK, but not MEK/ERK or PI3K-mediated signaling pathway. The phosphorylation level of ACTN 4 stimulated by EGF was significant higher than that of ACTN 1. The multiple phosphorylation sites of ACTN4 localized within the N-terminal 1-275 amino acids. The C-terminal 400-911 of ACTN4 acted as an inhibitory domain for actin binding and EGF-mediated phosphorylation. Binding of ACTN4 to actin prevented it from being phosphorylated upon EGF exposure. Compared to WT ACTN4, actin binding capacity and the susceptibility to the cleavage by m-calpain of ACTN4 mutants with phospho-

tyrosine mimics (Y260E and Y265E) were significantly enhanced. These data suggest that EGF-induced actinin phosphorylation is controlled by interactions with actin filaments.

1094/B302

Rac Regulates the Interaction of Fascin with Protein Kinase C in Cell Migration.

J. C. Adams[1], M. Parsons[2]; [1]Cell Biology, Cleveland Clinic, Cleveland, OH, [2]1Randall Division of Cell and Molecular Biophysics, King's College London, London, United Kingdom

Alterations in the expression or regulation of actin cytoskeletal components play an important role in the aberrant migration of carcinoma cells. Fascin is an actin-bundling protein that is low or absent in normal epithelia; its up-regulation correlates with poor prognosis in many human carcinomas. In mouse xenograft models, fascin contributes to tumour metastasis through its dual actin-bundling and active PKC-binding activities. Rac has been implicated as an upstream regulator of fascin-dependent colon carcinoma cell migration in vitro. We tested the hypothesis that Rac regulates the interaction of fascin with active PKC, by immunofluorescence, biochemical and FRET microscopy methodologies. Endogenous PKCγ, fascin and Rac1 colocalised at lamellipodial margins of migrating human colon carcinoma cells. Colocalisation of fascin and PKCγ depended on Rac activity, and inhibition of Rac decreased PKCγ activity in cell extracts but not in vitro. Fluorescence resonance energy transfer/fluorescence lifetime imaging microscopy uncovered that fascin and PKCγ interact in protrusions and filopodia of the migrating cells. Mechanistically, the interaction depended on phosphorylated fascin, active PKCγ and active Rac1, but not on active Cdc42. Regulation of the fascin/PKC complex by Rac was mediated in part by Pak. Elucidation of this novel pathway that regulates the fascin/PKCγ complex in migrating carcinoma cells suggests novel targets for therapeutic intervention in metastasis.

1095/B303

Sarcomeric Actin Organization is Synergistically Promoted by Tropomodulin, ADF/cofilin, AIP1, and Profilin in C. elegans Body Wall Muscle.

S. Yamashiro[1], E. A. Cox[2], D. L. Baillie[3], J. Hardin[2], S. Ono[1]; [1]Pathology, Emory University, Atlanta, GA, [2]Zoology, University of Wisconsin, Madison, WI, [3]Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada

Sarcomeric organization of thin and thick filaments in striated muscle is important for efficient generation of contractile forces. Sarcomeric actin filaments are uniform in their lengths and regularly arranged in a striated pattern. Tropomodulin caps the pointed end of actin filaments and is a critical regulator of sarcomere assembly. Here, we report unexpected synergistic functions of tropomodulin with enhancers of actin filament dynamics in *Caenorhabditis elegans* striated muscle. Pointed-end capping by tropomodulin inhibited actin filament depolymerization by ADF/cofilin in vitro. However, in vivo, depletion of tropomodulin strongly enhanced disorganization of sarcomeric actin filaments in ADF/cofilin mutants, rather than antagonistically suppressing the phenotype. Similar phenotypic enhancements by tropomodulin depletion were also observed in mutant backgrounds for AIP1 and profilin. These in vivo effects cannot be simply explained by antagonistic effects of tropomodulin and ADF/cofilin in vitro. Thus, we propose a model in which tropomodulin and enhancers of actin dynamics synergistically regulate elongation and shortening of actin filaments at the pointed end.

1096/B304

Contribution of Filamin A to the Mechanics of Melanoma Cells.

K. E. Kasza[1], F. Nakamura[3], T. P. Stossel[3], N. Wang[2], D. A. Weitz[1]; [1]SEAS, Harvard University, Cambridge, MA, [2]Dept. of Mechanical Science and Engineering, University of Illinois at Urbana-Champaign, Urbana, IL, [3]Translational Medicine Division, Dept. of Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA

Filamin A (FLNa) cross-links F-actin into orthogonal networks in the cortex and also forms a link between the cytoskeleton and the cell membrane. In vitro, a minimal cytoskeleton formed with purified F-actin and FLNa can reconstitute the unusual nonlinear elasticity of cells. However, FLNa's contribution to cell mechanics is unclear. We measure the materials properties and contractility of a melanoma cell line lacking FLNa (M2) and compare to a transfected subline stably expressing FLNa (A7). We find that loss of FLNa is associated with a strong decrease in cytoskeletal tension and as a result, leads to softer cells. To perturb cytoskeletal tension we grow cells on substrates of varying rigidity and find that loss of FLNa leads to a decrease in cellular substrate rigidity response. Our results are consistent with the picture that FLNa is required for build-up of internal myosin-generated tension within the actin cytoskeleton, which is required for stiffening of the cell.

1097/B305

Mapping Of Drebrin Binding Site on F-Actin by Chemical Cross-Linking.

E. E. Grintsevich[1], A. J. Ytterberg[1], M. Mikati[1], J. A. Loo[1,2,3], E. Reisler[1,2]; [1]Chemistry & Biochemistry, UCLA, Los Angeles, CA, [2]Molecular Biology Institute, UCLA, Los Angeles, CA, [3]Department of Biological Chemistry, UCLA, Los Angeles, CA

Drebrin A is a brain specific F-actin binding protein involved in organizing the dendritic pool of actin. Previous in vivo studies identified actin-binding domain of drebrin (DrABD, residues 233-317), which causes the same rearrangements in cytoskeleton as the full length protein. N-GST fused DrABD 233-317 binds to actin with a relatively low affinity (Kd = 7 uM). Shortening the sequence (233-300) does not affect its binding to F-actin significantly. CD spectrum of drebrin ABD, which is independent of pH within a range from 5.0 to 8.6, yields a secondary structure composition of 28% helix, 15% β-sheet, 57% turns and random coil. We mapped the binding site of drebrin ABD on F-actin by chemical cross-linking methods. Mass spectrometry analysis revealed that a zero length cross-linker, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), can link Gly 1 in the N-terminal part of the recombinant drebrin's fragment (233-317), containing extra GS, to glutamic acid 99 and/or 100 on actin. For further mapping, the modification of DrABD with Traut's Reagent was employed to introduce sulfydryl (-SH) groups on the surface of the fragment. This modified DrABD could be cross-linked to cysteine 374 on F-actin using MTS1 (5.4 Å). At the same time, cysteine 374 on an adjacent actin protomer was cross-linked with MTS1 to drebrin's native Cys 308, yielding a population of actin dimer with DrABD attached to two adjacent protomers. This set of data shows that drebrin's sequence 233-317 binds along two actin protomers. Our results provide structural insight into the previously observed competition for F-actin binding

between drebrin and 'side binding' proteins like a-actinin (which interacts with the actin sequences 95-125 and 360-372) and tropomyosin (N-terminus and residue 95 are involved).

1098/B306

Diverse Roles of Actin Side-Binding Proteins Required for Cell Division in the Fission Yeast Schizosaccharomyces pombe.

C. Skau[1], E. M. Neidt[1], A. Bestul[1], D. R. Kovar[1,2]; [1]Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL, [2]Biochemistry and Molecular Biology, University of Chicago, Chicago, IL

Assembly of a mature cytokinetic contractile ring in the fission yeast *Schizosaccharomyces pombe* requires multiple actin-associated proteins with complementary properties such as nucleation (formin Cdc12p) and filament side-binding (tropomyosin Cdc8p and fimbrin Fim1p). We are investigating the mechanisms by which the actin filament side-binding proteins tropomyosin and fimbrin contribute to assembly and maintenance of a dynamic contractile ring in fission yeast. Although tropomyosin Cdc8p inhibits actin filament elongation on its own, we have discovered that Cdc8p enhances formin Cdc12p-mediated actin filament assembly by (1) binding preferentially with low nM affinity to newly assembled ATP-actin, (2) doubling the elongation rate of Cdc12p-associated filaments, and (3) allowing Cdc12p-nucleated filaments to anneal end-to-end. In contrast, fimbrin Fim1p binds with approximately 600 nM affinity to both new ATP- and older ADP-actin filaments and has no obvious effect on formin Cdc12p-mediated actin filament assembly. Fim1p effectively bundles actin filaments in both the absence and presence of formin Cdc12p; bundles have both parallel and anti-parallel orientations. Surprisingly, Fim1p also drastically reduces the affinity of tropomyosin Cdc8p for actin filaments. Therefore, Fim1p might be required to maintain a dynamic contractile ring in the presence of filament stabilizing proteins, a critical feature of contractile ring assembly in fission yeast.

1099/B307

SH2B1β is a Novel Actin Cross-linker.

L. Rider, J. Lu, B. Brinley, M. Diakonova; Biological Sciences, University of Toledo, Toledo, OH

The widely-expressed SH2 domain-containing adapter protein SH2B1β was initially identified as a binding partner and substrate of JAK2 tyrosine kinase. SH2B1β is a member of the SH2B family (SH2B (SH2B1), APS (SH2B2), and Lnk (SH2B3)), which contains a conserved N-terminal dimerization, central pleckstrin homology, and C-terminal SH2 domains. Deletion of the SH2B1 gene results in severe obesity and both leptin and insulin resistance, as well as infertility, suggesting that SH2B1β may play a fundamental role in cell functions. SH2B1β has also been previously implicated in the regulation of the actin cytoskeleton by growth hormone and PDGF and cell motility. SH2B1β is also required for maximal actin-based motility of Listeria monocytogenes. SH2B1β increases the rate of bacteria propulsion in infected cells and in cell extracts in a VASP-dependent fashion. However, the mechanisms by which the effect of SH2B1β on the actin cytoskeleton are mediated remain unknown. Here we report that SH2B1β has two actin-binding sites. The first site contains amino acids 150-200 and binds F-actin with high affinity while the second site (amino acids 615-670) binds F-actin less strongly as assessed by high speed-cosedimentation assay. Using a low speed pelleting assay and electron microscopy, we showed that SH2B1β cross-links acin filaments in vitro. In cells, SH2B1β localizes not only to cell ruffles as previously shown but also along the filopodia. Deletion of amino acids 150-200 leads to mislocalization of the mutant protein to the filopodia "tip complexes" where the mutant co-localizes with VASP. Using VASP-deficient MVD7-/- cells and the same cells stably re-expressing GFP-VASP, we showed that proper intracellular localization of SH2B1β depends on the presence of the first actin-binding site and the presence of VASP. Finally, we demonstrate that both actin-binding domains of SH2B1β are required for maximal growth-hormone - induced cell ruffling and for cell motility as assessed by phagokinetic assay. We hypothesize that SH2B1β functions as an adapter protein that in response to JAK2 activation, cross-links actin filaments leading to modulation of cellular responses.

1100/B308

Toxoplasma gondii Actin Depolymerizing Factor Functions Primarily by Actin Monomer Sequestration.

S. Mehta, L. Sibley; Molecular Microbiology, Washington University School of Medicine, St. Louis, MO

Toxoplasma gondii is an obligate intracellular protozoan parasite belonging to the phylum Apicomplexa. Parasites in this phylum move by a unique process termed gliding motility, which requires transient assembly of parasite actin filaments beneath the parasite's plasma membrane. However, paradoxically parasite actin is predominantly monomeric or in small complexes. Treatment with jasplakinolide renders parasites hyperkinetic and unable to undergo directional motility, suggesting that rapid turnover of actin filaments is essential. ADF/Cofilin (AC) proteins are well-known for increasing actin filament turnover, and are one of few actin binding proteins conserved in Apicomplexans (sharing 30% identity with other AC proteins). Interestingly, homology modeling indicates that while Apicomplexan ADF's retain predicted G-actin binding sites, sites required exclusively for F-actin binding are absent. Here biochemical assays were used to examine the mechanism by which *T. gondii* ADF (TgADF) interacts with actin. In comparison to other AC proteins, TgADF caused almost complete filament disassembly in actin sedimentation assays, indicating a high net disassembly activity. However, in polymerization assays it exhibited a biphasic activity, enhancing the kinetics of actin polymerization at low ADF concentrations, while decreasing the rate and level of polymerization at high ADF concentrations. When steady-state assays were employed, TgADF inhibited steady-state actin polymerization even at high actin concentrations. Together these data suggest that at high concentrations, TgADF disassembles actin filaments by monomer sequestration. Mutation analysis is being used to determine if the lack of key F-actin binding sites accounts for this activity. AC proteins are typically thought to function via severing, pointed end depolymerization, and more recently, filament nucleation. Here we demonstrate that TgADF increases filament disassembly primarily by sequestering actin monomers. Whether this activity is specific to apicomplexan ADF's, or common to the ADF subset of AC proteins, awaits determination.

1101/B309

Actin Binding Proteins ADF and Profilin Control Gene Expression and Alter Multicellular Development.

R. B. Meagher, M. K. Kandasamy, E. C. McKinney, D. R. Ruzicka, L. M. King-Reid; Genetics, University of Georgia, Athens, GA

We are interested in the cytoplasmic and nuclear roles that conventional plant actin and actin binding protein (ABP) family members play in multicellular development. Our hypothesis is that *"Conventional actins and ABP isovariants provide a networked link between cytoplasmic cytoskeletal functions and nuclear epigenetic control"*. We localized a subset of *Arabidopsis* ADF/cofilin and profilin isovariants to the nucleus. Several *ACTIN DEPOLYMERIZING FACTOR- (ADF/COFILIN-)* and *PROFILIN*-mutant plants have developmental defects, including altered organ and cell morphology and cytoskeletal organization. The mutant phenotypes can be complemented by transgenes encoding homologous, but not heterologous isovariants in the same family. A subset of the ABP-defective developmental defects resembled those we observed in plants deficient in nuclear *ACTIN-RELATED PROTEINS (ARPs), ARP4, 5, 6,* and *7,* which are epigenetic factors controlling chromatin remodeling. Examples of developmental and molecular phenotypes implicating a possible role in *"nuclear epigenetic control"* include the following: *ADF3*- and *ADF9*-defective mutants that flowered early; profilin PRF1-defective mutants that flowered late, and ADF5-defective plants that were cold-resistant. The *ADF9-, ADF5-,* and *PRF1*-defective plants showed significantly altered expression of transcripts encoding high-level repressors and activators of the appropriate developmental pathways and some of these factors are known to be under epigenetic control. For example, in *ADF9*-defective plants the flowering time repressor *FLOWERING LOCUS C (FLC)* is down regulated, chromatin structure at *FLC* is more compact, and histones are differentially modified, while in *ADF5*-defective plants the cold protection activator *CBF1 (C-REPEAT/DRE BINDING FACTOR)* is upregulated and *CBF1* shows dramatically altered chromatin structure. Because ADFs and profilins may shuttle actin and other proteins between the cytoplasm and nucleus and alter the binding of regulatory proteins to F-actin in the cytoplasm, the ABP genes and isovariants described herein may provide *"a networked link"* between the two cellular compartments.

1102/B310

Tropomodulin /Tropomyosin Interactions as a Possible Mechanism of Tropomodulin Regulation.

Y. Gritsyna[1,2], A. Kostyukova[1]; [1]Neurobiology & Cell Biology, RWJMS-UMDNJ, Piscataway, NJ, [2]Institute of Protein Research, RAS, Pushchino, Russian Federation

Tropomodulin is an actin-capping protein for the pointed end of thin filaments. The mechanisms that regulate tropomodulin's function as a capping protein are still unknown. We hypothesize that isoform-specific tropomyosin/ tropomodulin interactions are a mechanism for fine-tuning pointed end dynamics. There are four tropomodulin isoforms encoded by separate genes: Tmod1, Tmod2, Tmod3, and Tmod4; their distribution in cells and tissues is different. For effective capping, tropomodulin needs tropomyosin. Tmod1 contains two binding sites for both short and long tropomyosin isoforms. Binding to the Tmod1 sites is isoform-specific. In this study we measured affinities of two other tropomodulin isoforms, Tmod2 and Tmod3, for tropomyosin model peptides using tropomodulin fragments, which contain one of two tropomyosin-binding sites. This method allows us to compare affinities of individual tropomodulin sites for different tropomyosin isoforms. The first and second tropomyosin-binding sites of Tmod2 and Tmod3 were localized by homology to the Tmod1 tropomyosin-binding sites and the corresponding synthetic peptides were ordered. The ability to form complexes was analyzed using native gel-electrophoresis, cross-linking and circular dichroism. Dissociation constants were calculated from circular dichroism melting curves and compared. Affinities of Tmod2 and Tmod3 for tropomyosin isoforms differ from those of Tmod1. While long α-tropomyosin binds well to Tmod1, its binding to Tmod2 and Tmod3 is weak. Short δ-tropomyosin binds better to Tmod2. We also analyzed sequences of regions corresponding to the first and second tropomyosin-binding sites in different isoforms of tropomodulin and leiomodin (a larger tropomodulin homolog). Based on the binding results residues that are not important for isoform tropomyosin-binding specificity were inferred. Together, tropomyosin- and tropomodulin-dependent specificity in binding may allow more variations in regulation of pointed end dynamics. Supported by American Heart Association Grant 0535328N and the UMDNJ Foundation Grant.

1103/B311

Alternate Splicing and Polymerization are Involved in Sorting Tropomyosin Isoforms to Actin Filaments.

C. Martin[1,2], G. Schevzov[1,2], P. Gunning[1,2,3]; [1]Oncology Research Unit, The Children's Hospital at Westmead, Sydney, NSW, Australia, [2]Discipline of Paediatrics and Child Health, The University of Sydney, Sydney, NSW, Australia, [3]Dept of Pharmacology, School of Medical Sciences, The University of New South Wales, Sydney, NSW, Australia

Tropomyosin (Tm) forms a head-to-tail polymer that runs along the actin filament and has multiple roles in regulating actin filament function. A large number of Tm isoforms are generated from alternative splicing of four genes. These isoforms are able to sort to different cellular compartments, and this sorting of Tm isoforms contributes to the spatial specialisation of actin filament function. Changes in Tm expression occur in cancer, with a trend towards cancer cells relying more on low molecular weight isoforms. This, along with sorting of Tms into different functional compartments, provides an attractive approach to target the actin cytoskeleton with chemotherapy while sparing the contractile systems of the heart and diaphragm. While Tm sorting has been observed in a number of cell types, there is little understanding of the mechanisms underlying this process. We are investigating sorting of different Tm isoforms using fluorescent-tagged Tm constructs and anti-Tm antibodies. We are also investigating the role of polymerization in Tm sorting via the characterisation of Tm mutants. There are differences in sorting between low molecular weight and high molecular weight αTm isoforms, which differ at their N-termini. Although both sets of isoforms localize to stress fibres, low molecular weight isoforms appear relatively enriched in peripheral regions of the cell compared to high molecular weight isoforms. This observation indicates that alternative exon choice at the N-terminus can influence sorting. Deletion of the N-terminal exons leads to a loss of sorting of these mutants to actin filaments. Deletion of the last 10aa of the C-terminus also leads to loss of sorting. We propose that polymerization and the ability to bind actin is a requirement for Tm sorting to actin filaments.

1104/B312

Tropomyosin 3 Inhibits the Ability of B35 Cells to Undergo Morphogenesis.

G. Schevzov[1,2], N. Bryce[1,2], R. P. Weinberger[1,2], P. W. Gunning[1,2,3]; [1]Oncology Research Unit, The Children's Hospital at Westmead, Sydney, NSW, Australia, [2]Discipline of Paediatrics and Child Health, The University of Sydney, Sydney, NSW, Australia, [3]Dept of Pharmacology, School of Medical Sciences, The University of NSW, Sydney, NSW, Australia

Tropomyosin (Tm) binds cooperatively to actin filaments and together with key actin binding proteins regulates their dynamics. A vast array of Tm isoforms, over 40, have been identified. Morphological alterations observed during development and cell differentiation correlate with changes in the expression of specific Tms. Tm isoform expression during differentiation of various cell systems including muscle, granulosa, lens and neuronal cells {Gunning et al 1990; Hughes et al., 2003; Ben Ze'ev et al., 1989; Fischer et al., 2000} results in reduced expression of the non-sarcomeric high molecular weight (HMW) Tms (Tm1, 2 and 3). The significance of this change in Tm isoform expression was investigated by maintaining the expression of a HMW Tm throughout differentiation. The rat neuroepithelial cell line, B35, can be induced to morphologically differentiate on exposure to dibutyryl cAMP (dbcAMP) and a concomitant downregulation of the HMW Tms (Tm1, 2, 3) and appearance of the neuronal specific TmBr3 is observed. In the B35 cell line stably transfected with the HMW Tm3 isoform, the persistence of Tm3 inhibited the ability of the cells to undergo morphological differentiation and fully exit the cell cycle. In contrast, in B35 clones overexpressing the brain specific Tm isoforms, TmBr1 and TmBr3, cellular morphogenesis proceeded following exposure to dbcAMP. A significant downregulation of the HMW Tms (Tm1, 2, 3) was observed and cell cycle arrest occurred as seen by a significant increase in the cells in G0-G1 and a decrease of cells in both S and G2-M phases. The data indicates that downregulation of the non-sarcomeric HMW Tms is required for normal morphological differentiation and complete withdrawl from the cell cycle. In the case of Tm3 expression, the mechanism involves locking the actin filaments into structures which cannot respond to the morphogenic signal. This can be reversed by pre-treatment with cytochalasinD.

1105/B313

Role of NHERF-1 in the Regulation of T lymphocyte Adhesion and Migration.

K. Ben Aissa[1], Y. Liu[1], I. Shomer[1], J. Hao[1], D. Steplock[2], E. J. Weinman[2], S. Shaw[1]; [1]EIB, NCI, Bethesda, MD, [2]University of Maryland, School of Medicine, Baltimore, MD

Cytoskeletal reorganization is critical during adhesion and migration of T lymphocytes. Some of the proteins that regulate these processes link the cytoskeleton to the plasma membrane. Of particular importance is the ERM (ezrin, radixin, moesin) family of proteins which are particularly enriched in the membrane extensions or microvilli. Other proteins able to provide such link remain to be identified. By mass spectrometry analysis we found that the protein that is most enriched in a microvilli-containing fraction of T lymphocytes (relative to post-nuclear lysate) is NHERF-1 (Na+/H+ exchanger regulatory factor-1). NHERF-1 is a scaffolding molecule that links membrane receptors to a variety of cytoplasmic signaling proteins such as kinases, phospholipases, and cytoskeletal proteins. Despite its abundance and its particular localization in microvilli, NHERF-1 role in T lymphocytes has not yet been studied. We therefore investigated immune system development and function in NHERF-1-knockout (KO) mice. Hematopoietic system development was found to be normal. However, alterations were observed in two specific in vitro cellular responses. Analysis of chemokine (SDF-1) induced transmigration assay showed that NHERF-1 KO T lymphocytes transmigrate about two times more efficiently than wild-type cells. Conversely over-expression of NHERF-1 (in Jurkat T-cells) inhibited SDF-1 induced transmigration. Spreading area and actin polymerization on CD3 coated surface are reduced in KO cells. Moreover the measurement of intracellular pH of KO cells shows that intracellular alkalinization during spreading is impaired. Our findings indicate that NHERF-1 participates in the regulation of T cell migration and spreading probably by regulating the actin reorganization during the adhesion phase of migration. The molecular mechanisms of the regulation of actin reorganization through NHERF-1 are under investigation.

1106/B314

Differential Proteomic Analysis of Cytoskeletons from HDAC6-null Cells.

A. Tran[1], S. R. Alaei[1], L. M. Brown[3], J. Bulinski[1,2]; [1]Biological Sciences, Columbia University, New York, NY, [2]Pathology and Cell Biology, Columbia University, New York, NY, [3]Comparative Proteomics Center, Department of Biological Sciences, Columbia University, New York, NY

The class II histone deacetylase, HDAC6, has been identified as a key regulator in a number of critical cellular processes, such as inflammation, viral infection, wound healing, and tumor metastasis. HDAC6 modulates the cytoskeleton both through its deacetylation of substrates such as tubulin, cortactin, and Hsp90, as well as through its interactions with PP1, mDia2, p150glued, and β-catenin. We have previously studied HDAC6 and its effects, through its deacetylation of microtubules, on focal adhesion turnover, vesicle trafficking, and cellular polarity. Since HDAC6 is involved in multiple pathways, we attempted an unbiased, systematic approach to identify all HDAC6-dependent cytoskeletal effects. Using a proteomic screen, we compared HDAC6-null mouse fibroblasts (HDAC6 KO MEFs) to their WT counterparts. Detergent-solubilized cytoskeletons were analyzed using Differential in Gel Electrophoresis (DIGE), wherein each WT and HDAC6 KO sample is separately labeled with a different fluorescent dye. Labeled cytoskeletons were then combined and the mixture was subjected to 2D PAGE. Changes in fluorescence intensities allowed direct comparison of the two samples within a single 2D gel. We detected a number of spots whose fluorescence intensity levels were altered by 1.5 to 10-fold. These spots, representing proteins under- and over-represented in cytoskeletons from HDAC6 KO cells, were then picked, trypsin digested, and identified by peptide mass fingerprinting. Proteins over-represented in HDAC6 KO cytoskeletons included the following: lamin A, septin 11, gelsolin, Hsp70 4L, and myosin light chain 6; under-represented proteins included: α-catenin and ezrin. The targets identified from this screen will serve as a foundation for future investigation into the role of HDAC6 in cytoskeletal regulation.

1107/B315

Thymosin B4 (Tβ4), a Major Intracellular G-actin Sequestering Protein, Demonstrates a Novel Anti-Inflammatory Function by Suppressing TNF-A-Induced NF-Kb Activation and the Production of Cytokines/Chemokines in Human Corneal Epithelial Cells.

P. Qiu, Y. Qiu, M. Kurpakus-Wheater, G. Sosne; Ophthalmology, Anatomy & Cell Biology, Wayne State University, Detroit, MI

Thymosin beta-4 (Tβ4), a G-actin sequestering peptide, has been found to be involved in physiological and pathological processes such as vasculogenesis and angiogenesis, wound repair, and tumor metastasis as well as modulation of the immune response. An increasing number of intracellular proteins targeted by Tβ4 have been identified, which suggests that interactions between Tβ4 and associated proteins provide the molecular basis for Tβ4's multiple functions. Using both primary (HCEC) and immortalized human corneal epithelial cells (HCET), we

conducted a study to explore the determining mechanism of Tβ4's anti-inflammatory function by interfering with tumor necrosis factor (TNF)-α-induced nuclear factor kappa B (NF-κB) activation and the production of cytokines/chemokines such as interleukin (IL)-8. Tβ4 significantly decreased the secretion of IL-8 protein from corneal cells following TNF-α stimulation either by ectopic treatment or enforced expression. In HCET, Tβ4 interfered with TNF-α signaling by suppressing NF-κB p65 expression and its translocation into nuclei as well as suppressing the phosphorylation of IκB kinase (IKK). In the presence or absence of TNF-α, enforced expression of Tβ4 in HCET significantly inhibited luciferase reporter activities driven by concatenated DNA elements identified by NF-κB p65 as well as IL-8 promoter. Overexpression of PINCH-1 and ILK, components of a complex which might function as Tβ4's intracellular receptor, sensitized TNF-α induced p65 binding activity and partially relieved the inhibitory effects of Tβ4. Overexpression of another major G-actin binding protein, profilin-1, alone did not affect TNF-α mediated p65 binding, but greatly increased the Tβ4's inhibitory effects when both proteins were overexpressed. Enforced expression of Tβ4 also antagonized the p65 binding activity mediated by its overexpression. Both wild-type and mutant of Tβ4 potentially lacking actin binding activity inhibited p65 function. Also, the treatment of HCET by cytochalasin D, which destroys polymerized actin fibers, could not inhibit TNF-α induced p65 binding activity. These findings demonstrate a novel mechanism of anti-inflammation of Tβ4 by differential targeting to intracellular partners.

1108/B316

Expression of Coronin-3 (Coronin-1C) in Diffuse Gliomas Is Related to Malignancy.

C. P. Xavier, C. Clemen, A. A. Noegel; Centre for Biochemistry, Institute of Biochemistry I, Medical Faculty, Cologne, Germany

Coronins are highly conserved but poorly understood proteins. They play a central role in various cellular processes including signal transduction, transcriptional regulation, remodelling of the cytoskeleton, and regulation of vesicular trafficking. Coronin-3, a homotrimeric F-actin binding protein, has been shown to be important for cell migration and brain morphogenesis. Here, we present for the first time a detailed analysis of the expression pattern of coronin-3 in human brain tumours and demonstrate that coronin-3 expression correlates with malignant phenotype in diffuse gliomas. In general, the expression of coronin-3 varies in different brain tumour entities. However, in diffuse gliomas, the number of coronin-3 expressing tumour cells correlates with the degree of malignancy. High-grade gliomas, such as anaplastic astrocytomas, anaplastic oligodendrogliomas, anaplastic oligoastrocytomas and glioblastomas, show high numbers of tumour cells positive for coronin-3, while diffuse low-grade gliomas, such as diffuse astrocytomas,oligodendrogliomas and oligoastrocytomas, exhibit low numbers of coronin3-positive tumour cells. In order to explore and verify a contribution of coronin-3 to the malignant phenotype of diffuse gliomas, we employed an efficient shRNA-mediated coronin-3 knockdown in U373 and A172 human glioblastoma cells. Coronin-3 knockdown glioblastoma cells exhibited reduced levels of cell proliferation, cell motility and invasion into extracellular matrix compared to control cells. Together, our findings demonstrate evidence for a contribution of coronin-3 expression in the malignant progression of diffuse gliomas.

1109/B317

Role of Rho GTPases in Phagocytic Signalling: a siRNA Approach.

G. Tzircotis, C. Escaron, E. Groves, A. Dart, E. Caron; Imperial College London, London, United Kingdom

Phagocytic uptake is a highly ordered and stringently controlled process. Driven by a local re-organisation of the actin cytoskeleton, it is orchestrated by signalling through the Rho GTPase family of small G proteins. The best-studied phagocytic receptors are the Fcγ Receptor (FcγR) and Complement Receptor 3 (CR3, aka the αMβ2 integrin). Although it is well documented that phagocytic signalling through each of these receptors occurs through a specific subset of Rho GTPases, previous studies have largely relied on data using dominant negative mutants and/or toxins which can simultaneously inactivate multiple Rho GTPases. In this study we have used a short interfering RNA (siRNA) approach to perform a comprehensive screen of Rho GTPase involvement in both FcγR and CR3-mediated phagocytosis. Our results show that several of the 22 Rho-family members regulate phagocytic signalling and knock-down of these was found to positively or negatively affect phagocytosis through FcγR or CR3. This approach confirmed the notion that several sets of Rho proteins control actin polymerization during phagocytic uptake from distinct phagocytic receptors. It also revealed exciting new insights as to the Rho-dependent basic mechanisms controlling phagocytosis in professional and non-professional phagocytes.

1110/B318

Cells Expressing a Predicted Caspase I Cleavage Product of T-plastin Produce Actin Inclusions.

D. Patrick, A. Maselli; Biological Sciences, Chicago State University, Chicago, IL

Inclusions composed of actin filaments and actin binding proteins (ABP) have been observed in a number of neurodegenerative diseases. Expression of truncated forms of two *Dictyostelium* actin binding proteins (ABP 34 and fimbrin) results in formation of actin inclusions. One possible mechanism for truncated ABPs to arise in cells is by protease cleavage. We hypothesize that a caspase I cleavage fragment of the human fimbrin homologue T-plastin will be capable of inducing actin inclusions in cells. Analysis of the T-plastin sequence predicts three cleavage sites. We report the results of expression of a GFP tagged fragment extending from the third caspase I cleavage site to the end the protein (aa 422-630) in *Dictyostelium*. This fragment contains a large region of the second actin binding domain. We have verified expression of this fragment by western blot of cell lysates. When we observe the GFP signal generated by expression of this tagged fragment we clearly see inclusions in the cytoplasm. The GFP signal from these inclusions clearly co-localizes with a rhodamine phalloidin signal indicating that these inclusions are rich in filamentous actin. Ultrastructural observation of these cells show regions of material consistent with other actin inclusions that we have observed. This work suggests a possible connection between ABP fragments produced from the cleavage activities of endogenous proteases and the formation of actin inclusions including Hirano Bodies. This work supported by NIH/NIGMS grant #S06GM008043.

Actin Dynamics & Assembly I (1111 – 1131)

1111/B319

Role of the Actin Nucleators Arp2/3 and hDia1 in Distinct NK Cell Functions.

B. Butler; William Butler, Washington University, Saint Louis, MO

Natural killer cells are innate immune cells involved in the recognition and elimination of aberrant cells, including virally infected cells or cells that have undergone partial de-differentiation. The process by which NK cells ultimately interact with a potential target cell is a highly coordinated and regulated process, involving extravasation, and migration to the site of infection, inflammation, or tumorigenesis. NK cells interact with potential target cells through multiple cell-surface receptors involved in the recognition of and interaction with multiple ligands on potential target cells. Depending upon the receptor/ligand repertoires of both the NK and target cells, NK cells establish either a tight, stable "lytic synapse," resulting in lysis of the target cell, or a transient synapse, resulting in release of the target cell with no lysis. These processes are driven by the regulation and dynamics of the actin and microtubule cytoskeletons. There are currently two major classes of actin nucleators that induce the polymerization of distinct types of actin filaments and networks; the actin-related protein-2/3 (Arp2/3) complex and the formins. In this study we have determined that Arp2/3 is critical for adhesion to ICAM-1 and subsequent actin assembly at the lytic synapse (LS). The loss of Arp2/3 expression does not however, disrupt LFA-1 activation, which is required for adhesion to ICAM-1. Arp2/3 is dispensable for adhesion to fibronectin, and chemotactic migration on fibronectin. Interestingly, while the formin hDia1 is required for NK-mediated cytolysis, its' effect is not mediated through the actin cytoskeleton but through the microtubule cytoskeleton as loss of hDia1 expression disrupts the lytic synapse localization of the microtubule binding proteins EB1 and APC thus leading to aberrant microtubule localization and subsequent loss of lytic granule release. Therefore, we have determined that the actin nucleators Arp2/3 and hDia1 have distinct functions in NK-mediated cytolysis.

1112/B320

Cell Cycle-Dependent Ubiquitination of the Mammalian Diaphanous-Related Formin mDia2.

A. D. DeWard[1,2], A. Alberts[1,2]; [1]Cell Structure and Signal Integration, Van Andel Research Institute, Grand Rapids, MI, [2]Program in Cell & Molecular Biology, Michigan State University, East Lansing, MI

The mammalian Diaphanous-related (mDia) formins are autoregulated GTPase effectors that nucleate and elongate nonbranched actin filaments. The three mDia (mDia1/2/3) formins have been shown to regulate cell migration, cell division, vesicle trafficking, tumor suppression, and microtubule stabilization. However, the specific mechanisms by which mDia formins contribute to each of these processes is only beginning to be realized. Based on previous observations in our laboratory, we hypothesized that mDia2 is post-translationally modified, and that its modification is required to carry out its normal cellular functions. We now show that 1) mDia2 is post-translationally modified by ubiquitin; 2) mDia2 is attached to a polyubiquitin chain; and 3) mDia2 polyubiquitination is cell cycle dependent. These studies point to important roles for the regulated modification of the formin mDia2 to mediate conserved cellular processes such as cell division.

1113/B321

Liprin-α Sequesters mDia Through Direct Binding and Critically Regulates Actin Polymerization in Cultured Cells.

S. Sakamoto[1], T. Ishizaki[1], C. Higashida[1], N. Watanabe[1], K. Ohkawa[2], S. Narumiya[1]; [1]Pharmacology, Kyoto University Faculty of Medicine, Kyoto, Japan, [2]Frontier Technology Center, Kyoto University Faculty of Medicine, Kyoto, Japan

mDia consisting of three isoforms is a member of Diaphanous-related formins that catalyze actin nucleation and polymerizaiton to produce unbranched actin filaments. mDia is composed of, from the N-terminus, the GTPase-binding domain (GBD), Armadillo repeat region (ARR), dimerization domain (DD), formin homology (FH)1 and 2 domains and Diaphanous autoregulatory domain (DAD). DAD and ARR bind each other to close and inhibit mDia. The GTP-bound form of Rho binds to GBD and disrupts this intramolecular binding to release auto-inhibition in vitro. However, whether this is the sole regulatory mechanism of mDia in the cell remains unknown. To examine this issue, we have used a pull-down assay with an N-terminal region of mDia1 fragment as a bait, screened proteins interacting with mDia1 in mouse brain supernatant, and identified Liprin-α3. Similar binding was also detected on Liprin-α1. Interaction between endogenous Liprin-α1 and 3 and endogenous mDia1 in brain supernatant was confirmed by co-immunoprecipitation. Deletion analysis of each of the two proteins revealed that the C-terminal half of the coiled-coil domain in the central region of Liprin-α3 and the ARR and DD of mDia1 are responsible for interaction. The corresponding region of mDia2 and mDia3 also bind to the mDia-interacting region of Liprin-α3. Gel filtration and in vitro pull-down assay of recombinant proteins of these regions confirmed the direct binding. The incubation of the mDia-interacting region of Lipirin-α3 with mDia1 concentration-dependently inhibits actin nucleation and polymerization activity of mDia in vitro. Consistently, overexpression of a Liprin-α3 truncation mutant in vivo in cultured HeLa cells reduces the Rho-mediated formation of stress fibers but not that induced by an active mDia1 mutant lacking the Liprin-α interacting domain. Conversely, depletion of Liprin-α enhanced the formation of actin bundles in HeLa cells as well as NIH 3T3 cells. These results suggest that Liprin-α functions as a negative regulator of mDia and regulates the extent of actin assembly in the cell.

1114/B322

The Formin FRL1 (FMNL1) Is Upregulated In Macrophage Differentiation and Localizes to Podosomes.

A. Mersich, S. Blystone; Cell & Developmental Biology, SUNY Upstate Medical University, Syracuse, NY

Macrophages interact with the ECM through dynamic adhesion structures termed podosomes. These actin rich structures interact with the ECM through members of the beta-2 and beta-3 integrin families. Previous studies have demonstrated that surface expression of beta-3 integrins is upregulated during monocyte differentiation to macrophages. Our lab has studied the downstream pathway from beta-3 integrin activation and recently demonstrated that inhibition of formin activity downstream of the integrin inhibited actin filament formation and adhesion in vitro. After five days in culture with GM-CSF, human peripheral blood monocyte-derived macrophages increase expression of FRL1 six-fold in a manner temporally similar to the increase in beta-3 integrin expression as determined by RT-PCR and Western Blotting. Using immunofluorescent microscopy we have found that FRL co-localizes with actin and beta-3 in macrophage podosomes. We then generated fluorescently-tagged constructs of full-length FRL1, FRL1-FH1FH2Cterm, FRL 1-392, and FRL 1-457. Using these constructs as well as fluorescently-tagged full-length and dominant negative Pyk2 and Vav1 along with pharmacological inhibitors of components of the

beta-3 signaling pathway, we have examined the relationship between the beta-3 integrin signaling pathway and FRL1 localization to the macrophage podosome.

1115/B323

Formin Differentially Utilizes Profilin Isoforms to Rapidly Assemble Actin Filaments.

E. M. Neidt[1], B. J. Scott[1], N. Younger[1], D. R. Kovar[1,2]; [1]MGCB, The University of Chicago, Chicago, IL, [2]BMB, The University of Chicago, Chicago, IL

Multiple formin isoforms are necessary to drive the assembly of profilin-actin for diverse cellular processes. Given that many organisms contain several profilin isoforms, specific formin/profilin pairs might be matched to optimally stimulate actin polymerization. We utilized a combination of 'bulk' actin polymerization and single filament evanescent wave fluorescence microscopy assays to measure the effect of different profilin isoforms on the actin assembly properties of the cytokinesis formins from fission yeast (Cdc12p) and the nematode worm (CYK-1). We discovered that Cdc12p only effectively utilizes the single fission yeast profilin isoform SpPRF. Conversely, CYK-1 prefers the essential worm cytokinesis profilin CePFN-1 to the two non-essential worm profilin isoforms (SpPRF = CePFN-1 > CePFN-2 > CePFN-3). Profilin isoform specificity extends to other formins as well. Mouse formins mDia1 and mDia2 prefer mouse profilin 1 over mouse profilin 2a. Chimeras containing the profilin-binding formin homology 1 (FH1) domain from one formin and the barbed-end associated FH2 domain from the other formin, revealed that both the FH1 and FH2 domains help confer profilin isoform specialization. Although the Cdc12p and CYK-1 FH1 domains cannot differentiate between profilin isoforms in the absence of actin, formin FH1 domains appear to preferentially select specific isoforms of profilin-actin. Furthermore, profilin isoform specialization is physiologically important because temperature sensitive fission yeast profilin mutants cannot be complemented by profilins that do not work with Cdc12p. Therefore, rapid formin-mediated elongation of profilin-actin depends upon favorable interactions of profilin-actin with the FH1 domain as well as the barbed end-associated FH2 domain. Specific formin FH1FH2 domains are tailored to optimally utilize actin bound to particular profilin isoforms.

1116/B324

A Role of Homeostasis between G- and F-Actin in Filament Nucleation and Actin Polymer Restoration by mDia1.

C. Higashida, N. Watanabe; Pharmacology, Kyoto University Faculty of Medicine, Kyoto, Japan

Current modern microscopy allows visualization of actin reorganization processes at the single-molecule level in living cells. Previously, we have elucidated the processive actin capping mechanism of Formins. FH2 and FH1-FH2 domains of mDia1 visualized as single-molecules show fast directional movement over long distance after nucleating a filament. This unique property allows observation of both actin nucleation and elongation by mDia1. Interestingly, we found that a low-dose G-actin sequestering drug, latrunculin B (LatB) rapidly activates mDia1 to initiate directional movement. The FH2 region of mDia1, the core domain for actin nucleation, is sufficient to respond to LatB. In contrast, LatB had only negative effects on mDia1FH2-catalyzed actin assembly in vitro. To resolve this discrepancy, we built a model to estimate the concentration change in the G-actin species based on the binding constants between actin, LatB and actin sequestering proteins. By incorporating the observed imbalance between actin assembly and disassembly induced by LatB, our simulation analysis reveals an unexpected rapid increase in drug-free G-actin. Actin nucleation by mDia1 is therefore prompted not only by Rho but also strongly through increased catalytic efficiency of the FH2 domain reacting to the G-actin increase. Frequent actin nucleation by mDia1 was found around sites of vigorous actin disassembly marked by mPlum-tagged AIP1, supporting the role of G-actin in the regulation of mDia1. Another Formin, FRL1, but not Arp2/3 complex, was also activated by low-dose LatB. We propose a homeostatic mechanism by which transient accumulation of G-actin works as a cue to augment actin nucleation by mDia1 to restore cellular actin polymers.

1117/B325

INF2 is an Endoplasmic Reticulum-Associated Formin.

V. Ramabhadran, E. Seth Chhabra, H. Higgs; Biochemistry, Dartmouth Medical School, Hanover, NH

Formin proteins are actin assembly factors that accelerate filament nucleation, and then influence elongation rate by remaining processively attached to the elongating barbed end. Mammals possess 15 distinct formin proteins, but the cellular functions of most of these formins are unknown. Previously, we have shown that INF2 is a unique mammalian formin in that it accelerates both polymerization and depolymerization. Depolymerization activity relies on an actin monomer-binding WH2 motif. Our objective in this study was to determine the cellular localization and function of INF2. We find that both endogenous INF2 and GFP-INF2 localize strongly to the endoplasmic reticulum (ER) in Swiss 3T3 cells. This localization depends on a C-terminal farnesyl group, but additional interactions are also required. Mutations to the WH2 motif that block depolymerization, cause the ER to collapse onto the nucleus, and causes a large accumulation of actin filaments around the collapsed ER. We also examined INF2 regulation. Many other formins are auto-inhibited by interaction between an N-terminal Diaphanous Inhibitory Domain (DID) and a C-terminal Diaphanous Auto-regulatory Domain (DAD). INF2's DID and DAD interact with 1 μM affinity, and this interaction blocks depolymerization. Surprisingly, however, the DID/DAD interaction does not inhibit actin assembly by INF2. **Conclusions-** Localization of INF2 to ER suggests a role for actin dynamics in ER function. INF2's unique regulatory properties suggest that other formins might also require additional interactions, besides DID/DAD to inhibit polymerization. **Methods-** Immuno-fluorescence microscopy and live cell fluorescence microscopy to examine INF2 in cells. LC-MS/MS to identify the farnesyl group on INF2. Pyrene-actin fluorescence polymerization assay and fluorescence anisotropy to study INF2 regulation biochemically.

1118/B326

A Novel Actin Assembly Pathway.

V. Okreglak, D. Drubin; MCB, University of California Berkeley, Berkeley, CA

Actin filaments are assumed to assemble exclusively from actin monomers. While actin oligomers can form filaments by annealing in vitro, evidence that annealing contributes to actin assembly in living cells has not been reported. Here we show that a pool of actin that is resistant to the monomer-sequestering drug latrunculin A contributes to filament assembly in vivo. We further show that Aip1p is important for establishment of normal actin monomer concentration in cells and that Aip1p is required to efficiently convert products of cofilin-dependant

actin filament disassembly from oligomers to monomers in vitro. In addition, aip1Δ mutant cells are more resistant to monomer sequestration by latrunculin A. In total, these results suggest that annealing of actin oligomers is a physiologically relevant actin filament assembly pathway in vivo, and identify Aip1p as a crucial factor for converting short actin oligomers to monomers during disassembly.

1119/B327

Dual Effect of Caldesmon on the Kinetics of Actin Polymerization.

R. Huang, Z. Grabarek, C. Wang; Muscle and Motility Group, Boston Biomedical Research Institute, Watertown, MA

Caldesmon (CaD) is an actin-binding protein that reversibly inhibits smooth muscle contraction. The non-muscle isoform of CaD typically co-exists with the microfilaments in the stress fibers of quiescent cells, but the phosphorylated form is also found at the leading edge of migrating cells, where dynamic actin filament remodeling occurs. We have studied the effects of the C-terminal 32 kDa fragment of CaD (H32K) on the kinetics of in vitro actin polymerization by monitoring the fluorescence of pyrene-labeled actin. Addition of H32K or its Erk phosphorylated analog caused either an inhibition or an acceleration of pyrene emission enhancement, depending on whether it was added before or after the onset of actin polymerization. The later the CaD fragment was added relative to the onset of polymerization the less inhibition and faster enhancement was observed. Surprisingly, despite the differences in the final level of pyrene fluorescence there were similar amounts of polymeric actin in all samples, as determined from the pellets obtained by high-speed centrifugation. Based on these results we conclude the following: The polymeric actin occurs in at least two different conformational states, to both of which can CaD bind. Binding of CaD blocks the transition between the two conformations by arresting the filament at the pre-transition state, but once the filament proceeds beyond this transition, CaD stabilizes the post-transition conformation. The observed pyrene fluorescence increase therefore reports a conformational change in actin, not the actin polymerization per se; and the apparent suppression of the pyrene-actin fluorescence enhancement reflects the conformational state of the filament before such a transition. We postulate that actin filaments at the pre-transition state are less stable than those at the post-transition state. Thus CaD exerts a dual effect on the actin filament during its assembly. Unphosphorylated CaD binds assembled filaments in stress fibers and maintains the cytoarchitecture, whereas phosphorylated CaD binds to nascent actin filaments at the cell leading edge and preserves the less-stable state to permit more dynamic reorganization of the actin cytoskeleton.

1120/B328

Clustering of VASP Actively Promotes Processive, WH2 Domain-Mediated Actin Filament Elongation.

J. Faix[1], D. Breitsprecher[1], A. K. Kiesewetter[1], J. Linkner[1], C. Urbanke[1], G. P. Resch[2], J. V. Small[2]; [1]Institute of Biophysical Chemistry, Hannover Medical School, Hannover, Germany, [2]Institute of Molecular Biotechnology, Austrian Academy of Sciences, Vienna, Austria

Enabled/vasodilator-stimulated phosphoproteins (Ena/VASP) are a structurally conserved family found in vertebrates, invertebrates and Dictyostelium cells. All members of the family share a conserved domain structure: An N-terminal EVH1 domain required for subcellular localization followed by a central proline-rich region necessary for interaction with profilin, and finally a C-terminal EVH2 domain mediating interactions with actin as well as the multimerization of the molecule. Vertebrates express three Ena-related proteins Mena, EVL and VASP, which were shown to localize to sites of active actin assembly including focal adhesions, stress fibers, the lamellipodial leading edge and filopodial tips. Filopodia fail to form in Dictyostelium mutants lacking the single VASP member and are markedly reduced in neuronal cells devoid all three Ena/VASP proteins, consistent with a conserved requirement of this protein family in filopodia formation in evolutionary distant organisms. Despite the relevance of Ena/VASP proteins for many additional actin-based processes such as cell migration, cell adhesion and the intracellular movement of pathogens such as Listeria, their exact molecular function is controversial. Using in vitro TIRF microscopy we show that both human and Dictyostelium VASP are directly involved in accelerating filament elongation by delivery of monomeric actin to the growing barbed end. In solution, DdVASP markedly accelerated actin filament barbed end elongation in a concentration-dependent, non-processive manner. This activity was surprisingly not affected by profilin but was inhibited by low concentration of capping protein. Most notably, reconstituted actin filament elongation became processive upon clustering of VASP on functionalized beads and became virtually insensitive to very high concentrations of capping protein. Based on these data, the in vivo analysis of VASP mutants and on an EM structure of the protein, we propose a mechanism by which the flexible VASP tetramers employ their WH2 domain related actin-binding motifs to promote filament tethering to membranes during their processive elongation.

1121/B329

A Minimal Biomimetic System Demonstrating the Mechanochemical Function of Cortactin.

O. Siton[1], Y. Ideses[1], A. Bershadsky[2], A. Bernheim[1]; [1]Ben-Gurion University, Beer-Sheva, Israel, [2]Weizmann Institute of Science, Rehovot, Israel

Cortactin, a regulator of the Arp2/3 complex, is involved in invadopodia and podosome formation, pathogens and endosome motility, and persistent lamellipodia protrusion; its overexpression results in enhanced cellular motility and metastatic activity. Cortactin interacts with actin, the Arp2/3 complex, and WASP and WAVE, as well as with a number of regulatory and signaling proteins. Even though several mechanisms have been proposed to explain the possible role of cortactin in Arp2/3-dependent actin polymerization, the direct role of cortactin in cell movement and protrusion formation remains unclear. In a biomimetic in vitro motility assay, bead movement reproduced motility driven by Arp2/3-WASP (VCA domain)-induced actin polymerization. Cortactin localized primarily in the vicinity of the VCA-coated bead surface, but eventually translocated backwards, distributing itself over the entire "comet tail," in proportion to the actin density. Cortactin changed the morphology of the comet tail, apparently interfering with the depolymerization process. Moreover, cortactin significantly increased the rate of bead movement. Although cortactin alone could not promote Arp2/3-driven motility, it strongly augmented it, working in concert with VCA.

1122/B330

Cellular Localization and Expression of Eps8 (Epidermal Growth Factor Receptor Pathway Substrate 8) in Adult Rat Testis: It's Role in Junction Dynamics during Spermatogenesis.

P. Lie[1,2], D. D. Mruk[1], W. M. Lee[2], C. Cheng[1]; [1]The Population Council, New York, NY, [2]School of Biological Sciences, The University of Hong Kong, Hong Kong, China

Throughout spermatogenesis, extensive restructuring of cell junctions occurs at the inter-Sertoli and the Sertoli-germ cell interfaces. Since junction restructuring events are intimately related to the underlying actin cytoskeleton, we sought to investigate the role of Eps8 in these events. Eps8 is a 97 kDa protein that participates in multiple levels of actin regulation, including actin barbed-end capping, actin bundling, and the activation of the small GTPase Rac. By RT-PCR and immunoblotting, Eps8 and several of its binding partners, Sos-1, Abi1 and IRSp53, were shown to express in Sertoli and germ cells at relatively different levels. Eps8 was localized in adult rat testes by immunohistochemistry and fluorescent microscopy, showing a stage-specific expression pattern. At the site of the blood-testis barrier (BTB), Eps8 was highly expressed in stages V and VI tubules. The localization of Eps8 at the BTB was confirmed by its co-localization with ZO-1 and F-actin. Eps8 was also found at the apical ES, surrounding the heads of early elongating spermatids. The staining, however, shifted to the concave side of spermatid heads in stage VII tubules, and virtually disappeared prior to spermiation at late stage VIII. Following treatment of adult rats with adjudin (50 mg/kg b.w., by gavage) to induce anchoring junction restructuring in the testis, disappearance of Eps8 at the apical ES was detected in 'departing' germ cells by immunostaining, which was verified by a significant decrease of Eps8 protein by immunoblot analysis. Preliminary experiments were performed to study the function of Eps8 in primary Sertoli-cell cultures with established BTB that mimicked the tight junction barrier in vivo. Eps8 was transiently knocked down by using specific siRNA versus control duplexes, resulting in apparent anomalies in actin filaments, which include stress fiber truncation and a loss of organization. In summary, Eps8 is an important actin dynamics regulator in Sertoli cells for the maintenance of the BTB function.

1123/B331

AMP-Dependent Kinase is an Upstream Regulator of RhoA and Rac1 GTPase Activity and Cytoskeletal Organization during ATP-depletion in a Cell Culture Model of Ischemic Renal Injury.

H. Zhang[1], M. Hallett[2], S. Atkinson[2,1]; [1]Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, IN, [2]Department of Medicine Division of Nephrology, Indiana University School of Medicine, Indianapolis, IN

Knowledge of the molecular and cellular mechanisms of ischemic injury is necessary for understanding acute kidney injury and devising optimal treatment regimens. The cortical actin cytoskeleton in the proximal tubule epithelial cells of the nephron, playing an important role in both the establishment and maintenance of cell polarity, is drastically disrupted by the onset of ischemia. We have shown previously that dysregulation of the Rho GTPase signaling pathways is a critical mediator of the effects of ATP depletion and ischemia on the actin cytoskeleton, but the mechanism by which ATP depletion leads to altered RhoA and Rac1 activity is unknown. The AMP-dependent kinase (AMPK) is a key metabolic regulator that senses the cellular ATP:AMP ratio. Renal ischemia in vivo has been shown to activate AMPK. We propose that ischemia and ATP depletion result in activation of AMPK and that this affects Rho GTPase activity and cytoskeletal organization via decreased activity of mTORC2 and/or altered TSC1/2 activity. To test this hypothesis we used S3 epithelial cells derived from the proximal tubule in mouse kidney in a cell culture model of renal ischemia using antimycin A and substrate depletion to induce ATP depletion. We used phosphorylation of T172 of the AMPK α-subunit as a measure of AMPK activation and pull-down assays to measure the activity of RhoA and Rac1 GTPases. AMPK was rapidly activated (<5 minutes) by ATP depletion, and there was a corresponding decrease in RhoA and Rac1 activity. We used graded concentrations of glucose in the nutrient-depleted growth media containing antimycin A to achieve intermediate levels of ATP depletion, confirmed by HPLC analysis. We found intermediate levels of AMPK activity at these intermediate ATP levels while RhoA and Rac1 activity correlated inversely with the activity of AMPK. Activation of AMPK using metformin in S3 cells under normal growth conditions also suppressed RhoA activity, consistent with our hypothesis. We are further investigating this pathway using siRNA knockdown of the catalytic α-subunit of AMPK.

1124/B332

Cell Body Retraction in the MSP-based Motility of Ascaris Sperm is Initiated by Protein Phosphatase 2A.

K. Yi[1], M. Stewart[2], T. M. Roberts[1]; [1]Dept of Biological Science, Florida State University, Tallahassee, FL, [2]MRC Laboratory of Molecular Biology, Hills Rd., Cambridge CB2 2QH, United Kingdom

Amoeboid cell motility depends on coordination of leading edge protrusion with cell body retraction. In the crawling sperm of Ascaris, lamellipodial protrusion is driven by localized assembly of the MSP cytoskeleton, whereas cell body retraction is associated with cytoskeletal disassembly at the base of lamellipod. Reconstitution of retraction in cell-free extracts of Ascaris sperm, whereby cylindrical meshworks of MSP filaments (fibers) shorten when treated with selected agents, has allowed us to explore the biochemical basis of this essential component of sperm motility. Here, we report that PP2A, an evolutionarily conserved ser/thr phosphatase, triggers retraction of MSP fibers by dephosphorylation of MSP fiber protein 3 (MFP3), a structural component in the MSP motility apparatus. Perfusion of fibers with a commercial PP2A induced rapid shortening, or retraction, that was blocked by okadaic acid, a PP2A-specific inhibitor. Anti-PP2A antibody labeled a 34-kDa protein on Western blots of sperm extracts, which we confirmed as PP2A by mass spectroscopy. This sperm PP2A, purified by immunoprecipitation, induced okadaic acid-sensitive retraction when perfused onto fibers. In sperm, PP2A is concentrated at the junction of the lamellipod and cell body, where retraction occurs. PP2A selectively dephosphorylated MFP3, a unique 47-kDa protein found only in nematode sperm, that is phosphorylated and incorporated into the MSP filament meshwork during fiber assembly. Dephosphorylation of MFP3 by PP2A coincided with its release from fibers during retraction. Immunogold labeling showed that phospho-MFP3 binds to MSP filaments. Dephosphorylation by PP2A released MFP3 from MSP filaments and increased the rate of filament depolymerization. Thus, our results argue that PP2A triggers retraction by dephosphorylation of filament-bound MFP3, which, in turn, enhances cytoskeletal disassembly. In actin-rich cells, retraction appears to be powered by myosin. By contrast, MSP fiber retraction is not assisted by motor proteins and, instead, is initiated by dephosphorylation and destabilization of the motility apparatus. Supported by NIH Grant R37 GM 29994.

1125/B333

Dynamics of Actin Cytoskeleton Restoration after Blebbistatin Treatment.

M. Shutova[1,2], T. Svitkina[2]; [1]Carcinogenesis, Blokhin Cancer Research Center, Moscow, Russian Federation, [2]Biology, University of Pennsylvania, Philadelphia, PA

Contractile stress fibers and their anchorage sites, focal contacts, depend on each other for their assembly. However, it remains unclear how this cycle begins. Blebbistatin (BS), an inhibitor of the motor activity of myosin II (myoII), causes reversible disassembly of stress fibers in nonmuscle cells. We used light and electron microscopy (EM) to investigate how stress fibers and focal contacts are restored in REF52 fibroblasts after BS washout. At 50-75 uM, BS nearly completely disrupted stress fibers and mature focal contacts in the cell lamella, but dot-like focal complexes remained and could be suppressed only by 100 uM BS. In this case, lamellipodia were changed to irregular ruffling and numerous filopodia. BS treatment caused disassembly of myoII bipolar filaments, as revealed by the loss of the cytoskeleton-associated myoII and by EM of gelsolin-treated cytoskeletons. It suggests that assembly and/or maintenance of myoII filaments depend on the myoII motor activity, possibly, through tension-dependent regulation. Although the level of myosin light chain phosphorylation seemed unaffected by BS, the double phosphorylated (Thr18/Ser19) myoII became concentrated at the active cell edges, and not distributed all over the cell, as in untreated cells. The recovery from 100 uM BS began with rapid (within 1 min) formation of focal complexes at the active edges, while no obvious changes in myoII or actin bundle organization were detected by light or EM at this time. MyoII filaments became apparent only after 5 min of recovery. Simultaneously, thin disordered actin bundles containing myoII filaments were formed throughout the cell, while focal complexes increased in the number and size. Subsequent recovery consisted of gradual and coordinated maturation of focal contacts and stress fibers. We propose that myoII is activated by phosphorylation at the leading edges and initiates focal complexes there by pulling on actin filaments even before it visibly assembles into bipolar filaments. These initial adhesion sites support the subsequent stress fiber assembly, which occurs when myoII assembles into filaments and begins to orient and cross-link actin filaments into bundles. Supported by UPenn Research Foundation.

1126/B334

Myosin II Contributes to Cell Motility by Promoting F-Actin Disassembly near the Cell Trailing Edge.

C. Wilson[1], M. A. Tsuchida[1], G. Allen[1], E. Barnhart[1], K. Applegate[2], P. Yam[1], L. Ji[2], K. Keren[3], G. Danuser[2], J. Theriot[1]; [1]Department of Biochemistry, Stanford University School of Medicine, Stanford, CA, [2]Department of Cell Biology, The Scripps Research Institute, La Jolla, CA, [3]Department of Physics, Technion - Israel Institute of Technology, Technion City, Israel

Various types of crawling cells have been shown to exhibit defects in trailing edge retraction when myosin II activity is deficient. It has been suggested that the role of myosin II in rear retraction depends on its ability to contract the actin filament network, analogous to its role in muscle. We have found that, in fish keratocytes crawling at steady state, contraction does not play a significant role in rear retraction or whole cell movement. By tracking actin filament movement in live cells, we observed loss of network contraction upon inhibition of myosin activity using blebbistatin, without disruption of whole-cell movement. However, simultaneous inhibition of myosin activity and actin disassembly (using jasplakinolide) completely abolished cell movement, though inhibition of actin disassembly alone did not have this effect. Furthermore, the pattern of actin network disassembly in moving keratocytes correlated with the pattern of myosin II localization, and was sensitive to inhibition of myosin activity. In Triton-extracted keratocyte cytoskeletons, addition of ATP triggered both contraction and rapid loss of filaments. Taken together with a recently published report stating that purified myosin II can disassemble actin filaments in vitro without any other proteins (Haviv et al., 2008, J. Mol. Biol. 375: 325), our results suggest that myosin II activity promotes actin network disassembly at the cell trailing edge during cell motility, and that network disassembly rather than contraction is myosin's primary contribution to rear retraction and cell movement.

1127/B335

Divergent Unstable Actin in Apicomplexan Parasites is Rescued by Phalloidin.

K. M. Skillman[1], K. Tang[1], K. Diraviyam[2], D. Sept[2], L. D. Sibley[1]; [1]Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, MO, [2]Department of Biomedical Engineering, Center for Computational Biology, Washington University in St. Louis, St. Louis, MO

Plasmodium falciparum and Toxoplasma gondii are important parasitic pathogens of the phylum Apicomplexa. Parasite invasion of host cells involves a unique gliding motility mechanism that is dependent on polymerization of parasite actin. However, in non-motile parasites, the majority of actin is apparently monomeric and filaments only assemble upon initiation of gliding motility. Actin filament turnover is known to be crucial for motility as shown by the paralyzing effects of jasplakinolide, an agent that stabilizes actin filaments. T. gondii actin (TgACT1) is functionally highly divergent from conventional actin and only polymerizes into very short filaments. Remarkably, TgACT1 has a low critical concentration, but apparently fails to fully anneal and is thus unstable. P. falciparum, has two actin alleles, PfACTI and PfACTII, which are similar to TgACT1 and also diverge from conventional actin. The question remains as to why Toxoplasma and Plasmodium actins are unstable and what contributes to their unusual polymerization properties. We demonstrated that phalloidin can bind to parasite actins and is capable of rescuing the short filaments, as visualized by fluorescence microscopy and electron microscopy. In addition to the phenotypic impact of phalloidin on parasite actins, the kinetics of polymerization were also increased in the presence of phalloidin. These results demonstrate that although the parasite actins only form short, unstable filaments on their own, they are capable of forming longer, conventional filaments. Molecular modeling was used to identify residues unique to apicomplexan actin that may affect filament stability. These studies reveal critical substitutions in the hydrophobic plug, an intermolecular salt bridge, and the ATP-binding pocket in parasite actins. We have made mutations within TgACT1 that replace residues unique to apicomplexan actins with those from yeast actin in order to determine if these substitutions stabilize the TgACT1 filaments. These studies should reveal important structural and functional differences in divergent actins.

1128/B336

An RNAi Screen Identifies Host Cytoskeletal Proteins Required for Rickettsia Actin-Based Motility.

A. Serio, R. Jeng, C. Haglund, S. Reed, M. Welch; Molecular Cell Biology, University of California, Berkeley, CA

Spotted-fever group *Rickettsiae* are obligate intracellular pathogens that manipulate host cytoskeletal proteins to promote actin-based motility and comet-tail formation, facilitating cell-to-cell spread. The organization of actin filaments in *Rickettsiae* tails differs from that of other motile intracellular pathogens, suggesting that they utilize a distinct mechanism of actin assembly. To identify host proteins that function in *Rickettsia parkeri* actin-based motility, we targeted 107 cytoskeletal proteins in *Drosophila* S2R+ cells using RNAi, and quantified the effect on tail formation and the number of bacteria per cell. Twenty-six proteins were identified whose silencing resulted in a significant reduction in comet-tail formation and/or infection when compared to untreated controls. These included proteins that function in: 1) Arp2/3 complex-mediated actin nucleation, 2) actin monomer recycling, 3) actin bundling, 4) endocytosis and membrane-cytoskeletal interactions, 5) myosin-mediated force generation, 6) and cytoskeletal signaling. The most striking phenotypes were observed after silencing profilin, an actin-monomer-binding protein that promotes actin assembly, and fimbrin, an actin bundling protein. In profilin-silenced S2R+ cells, *Rickettsiae* failed to move in a directed fashion or to form actin comet tails, whereas in profilin-I-silenced mammalian cells, comet tails were reduced 5-fold in length. In fimbrin-silenced S2R+ cells, comet tail length was reduced but motility was unaffected, while in both fimbrin-silenced S2R+ and plastin (mammalian fimbrin)-silenced mammalian cells the number of comet tails was greatly reduced. In further support of an important role for plastin in mammalian cells, we found that GFP-plastin was localized to *Rickettsia* comet tails. Importantly, silencing of profilin or fimbrin/plastin in mammalian cells had no affect on *Listeria monocytogenes* comet-tail formation, verifying that these proteins are specifically important for *Rickettsia* actin-based motility. Through further study, we aim to identify the complete set of host proteins required for *Rickettsia* actin-based motility and to elucidate the molecular mechanisms of bacterial actin assembly and organization.

1129/B337

A Robustly Wrong Listeria Motility Model and Its Redemption --Agent-Based Modeling as a Method for Understanding Complex Biological Systems.

J. Alberts[1], S. M. Rafelski[2], G. M. Odell[1]; [1]Center for Cell Dynamics, University of Washington, Friday Harbor, WA, [2]Biochemistry, UCSF, San Francisco, CA

We combine agent-based simulations of L. monocytogenes motility with new experimental results on ActA distribution patterns to determine the dominant force mechanisms regulating bacterial speed. Populations of L. monocytogenes moving in the same extract system exhibit great variability in their steady-state speed, and some of this variability can be explained by differences in the surface distribution of ActA protein. By manipulating bacterial growth conditions, we extend the natural range of variability to create severely ultrapolar ActA distributions. Measurements of speed as a function of both ActA quantity and distribution constrain the model and suggest that a cooperative restraining mechanism -friction between the actin gel and the bacterial surface—largely determines this speed. This force has been ignored in all past L. monocytogenes motility models. This study also highlights two important features of our agent-based methodology, robust qualitative behavior and ability to adapt with our biological understanding. We encode biological interactions at the nanoscale and find that the microscale model behavior is qualitatively robust -this behavior was in our case robustly wrong until we introduced a crucial restraining mechanism. Recently revealed cooperativity of ActA proteins at sufficient density suggests an alternate plausible explanation of our experimental observations. We adapt the same model to incorporate this new biological information and explore (and ultimately discount) that hypothesis.

1130/B338

F- and G-Actin Concentrations in Lamellipodia of Moving Cells.

S. A. Koestler[1], K. Rottner[2], F. Lai[2], J. Block[2], M. Vinzenz[1], J. Small[1]; [1]Institute of Molecular Biotechnology, Austrian Academy of Sciences, Vienna, Austria, [2]Cytoskeleton Dynamics Group, Helmholtz Centre for Infection Research, Braunschweig, Germany

Cells protrude by polymerizing monomeric (G) into polymeric (F) actin at the tip of the lamellipodium. Actin filaments are depolymerized towards the rear of the lamellipodium in a treadmilling process, thereby supplementing a G-actin pool for a new round of polymerization. In this scenario the concentrations of F- and G-actin are principal parameters, but have hitherto not been directly determined. By comparing fluorescence intensities of bleached and unbleached regions of lamellipodia in B16-F1 mouse melanoma cells expressing GFP-actin, before and after extraction with Triton, we show that the ratio of F- to G-actin is 3.1+/- 0.9. Using electron microscopy to determine the F-actin content, this ratio translates into F- and G-actin concentrations in lamellipodia of approximately 500 µM and 160 µM respectively. The excess of G-actin, at several orders of magnitude above the critical concentrations at filament ends shows that polymerization is not diffusion limited and is tightly controlled by polymerization/depolymerization modulators.

1131/B339

The GEF/GAP Abr Regulates Rho and Cdc42 Activity Zones During Single Cell Wound Healing.

E. Vaughan, W. Bement; Zoology, University of Wisconsin, Madison, WI

During *Xenopus laevis* oocyte wound healing, organization of an acto-myosin contractile ring is controlled by zones of active Rho and Cdc42, which form concentric rings around wounds. We are now investigating how the activity of these GTPases is regulated during wound healing by GEFs, guanine nucleotide exchange factors, which activate the GTPases and GAPs, or GTPase activating proteins, which inactivate GTPases. To determine which GEFs and GAPs are involved in wound healing, we developed a functional screen based on localization and the effects on the Rho and Cdc42 activity zones of overexpression and dominant negative expression. Of the various GEFs and GAPs tested in this manner, only one, Abr, produced positive results in all 3 assays. Abr is a relatively uncharacterized GTPase regulator that contains a GEF domain as well as a GAP domain, and has been shown *in vitro* to act as a GEF for Rho, Cdc42, and Rac and a GAP for Cdc42 and Rac. Live imaging of GFP-Abr as well as immunofluorescence revealed localization of Abr to the zone of active Rho at the wound edge. When overexpressed, Abr eliminated the zone of active Cdc42, but upregulated the zone of active Rho. AbrΔGAP, where the entire GAP domain was deleted, failed to localize properly to the wound edge. However, AbrRN/AA, where residues critical for GTP hydrolysis were mutated, was able to localize to the zone of active Rho. Interestingly, the zones of active Rho and Cdc42 were suppressed relative to controls with these mutants. AbrΔDH, where the DH domain was removed, also failed to properly localize to the wound. Similarly, AbrNE/AA, with mutations in the DH domain important for binding GTPases, was unable to recruit to the wound. Overexpression

of these mutants resulted in diminished Rho activity with respect to controls. These data suggest that Abr may act to provide spatial segregation of the Rho and Cdc42 zones, positively regulating Rho and negatively regulating Cdc42. Furthermore, localization results suggest that Abr is recruited to the wound via binding to its substrates, Rho and Cdc42.

Centrosomes I (1132 – 1149)

1132/B340

Centrobin Mediates Centriole Duplication via Its Interaction with α-Tubulin and γ-Tubulin.

R. R. Gudi, C. Zou, Q. Gao; Medicine, Evanston Northwestern Healthcare Research Institute, Evanston, IL

Centrosomes constitute of two centrioles that duplicate once every cell cycle semi-conservatively. However, the process of centriole duplication still remains enigmatic. Previously, we have shown that centrobin asymmetrically localizes to the daughter centriole and is required for centriole duplication. In this study, we found that overexpression of centrobin results in the formation of bundle-like structures that colocalize with α- and γ-tubulins. Electron microscopy revealed an alternate electron dense and light pattern in these structures that resemble the paracrystals of lamins. Immunogold staining with centrobin suggests that these structures consist of organized centrobin filaments, which is further corroborated by our finding that full length centrobin has the potential to dimerize or polymerize. Furthermore, centrobin directly interacts with the major components of the centrioles, α- and γ-tubulin, both in vivo and in vitro through its C-terminal residues 765-903. This region of centrobin also contains the centrosome localization ability. And expression of truncated centrobin fragment (765-903) in mammalian cells blocks centriole duplication, likely via inhibiting the centrobin-tubulin interaction. We speculate that these centrobin filaments through their capacity to bind with tubulin act as scaffold during the centriole duplication, which are disassembled when the nascent centrioles become mature.

1133/B341

What Does Evolution Tell Us About Centriole Duplication?

Z. C. Santos[1], J. Pereira-Leal[2], M. Bettencourt-Dias[1]; [1]Cell Cycle Regulation, Instituto Gulbenkian de Ciência, Oeiras, Portugal, [2]Computational Genomics, Instituto Gulbenkian de Ciência, Oeiras, Portugal

Centrioles organize the centrosomes and form the basal bodies, which nucleate the axoneme of cilia and flagella. These structures are present in every branch of eukaryotes, suggesting a common origin in the ancestral eukaryote and a "core" conserved molecular mechanism for centriole biogenesis. We used comparative genomics and phylogenetic analysis to study the emergence and evolution of proteins known to be involved in centriole assembly. Since centriolar proteins are enriched in coiled-coils, which make automatic orthology assignment very difficult, we used sensitive search methods, developed classification criteria for each protein, and combined these with detailed phylogenetic analysis for orthology assignments. We then mapped new putative orthologs of these proteins throughout the eukaryotic tree of life. We clustered those proteins according to their phylogenetic profile. Our study revealed the presence of 3 centriole assembly molecular modules, generating new hypotheses regarding the molecular toolkit used to make centrioles,. A "structural core module", composed by SAS-4, SAS-6 and bld10/CEP135, correlates with the presence of the centriolar structure throughout the eukaryotic tree of life. A "contextual module", composed of SPD-2 and CP110, which only appeared late in evolution, in Metazoans/Choanoflagellates, indicating that new functions, or requirements for the assembly of the structure, might have been added later. Finally, we looked at kinases known to play a role in centriole assembly. We show that SAK/PLK4 appeared late in evolution, in the ancestor of fungi and animals suggesting that SAK/PLK4 central role was previously fulfilled by another kinase, perhaps the founder of its family, Polo. The appearance of SAK/PLK4 may be associated with linking centriole duplication to other aspects of centriole and cellular physiology. We are now testing some of those predictions using Drosophila as a model organism.

1134/B342

Characterization of Centrosome De-Novo Assembly in Vertebrate Cells.

J. Loncarek, T. Vinogradova, P. Hergert, A. Khodjakov; Molecular Medicine, Wadsworth Center, Albany, NY

In typical somatic cell, under normal conditions, daughter centrioles form in association with mature mother centrioles. However, centrioles can also assemble de novo. Although the de novo pathway has been shown to exist in a wide variety of vertebrate cell types, it is inhibited in the presence of resident centrosomes. The mechanism of this inhibition is unknown. As the first step toward revealing the interrelations between centriole duplication and de novo assembly pathways we seek to characterize details of centriole de novo assembly in cultured vertebrate cells. Our major approach is activation of de novo pathway by destroying the resident centrosomes via laser microbeam in cells where major centriolar proteins have been depleted or overexpressed. That will allow us to determine at which stage the process of centriole de novo formation will be impaired. The approach will also enable us to directly compare two modes of centriole assembly (duplication and de novo) in parallel. We are currently focusing on the roles of several proteins that have been shown to be essential for centriole duplication: Plk4, hsSAS6, and CPAP. We find that depletion of Plk4 completely blocks activation of the de novo pathway, consistent with the pivotal role of this kinase in centriole duplication. Depletion of hsSAS6 or CPAP prevents de novo assembly of morphologically-recognizable centrioles. However, cells depleted of these proteins still form centrin aggregates that manifest initiation of the de novo assembly. Thus, hSAS6 and CPAP participate in the de novo pathway downstream from Plk4. We also investigate roles of microtubule network and microtubule motors in centriole de novo assembly. Our preliminary data suggest that neither dynein-mediated transport nor even microtubules are required for the formation of centrin aggregates. Structural analyses are currently underway to determine if these centrin aggregates can develop into complete centrioles.

1135/B343

Laser Ablation of the Centrosome in Directionally Migrating Cells.

N. Wakida[1], V. Garces[1], L. Shi[2], E. Botvinick[1], M. Berns[1,2]; [1]Beckman Laser Institute, Irvine, CA, [2]UC San Diego, La Jolla, CA

Laser ablation is a useful tool in the study of biological processes such as cell migration. It allows for the controlled removal of targeted cellular structures in a living cell, without affecting surrounding intracellular organelles or the cells health. Here, we use a 200 femtosecond pulse duration near infrared laser to target the centrosome in human lung fibroblasts cells expressing GFP-centrin. This short-pulsed laser results in a small diameter of ablation down to 0.5 microns, and thus minimal off-target effects to the targeted cell. The laser microscope system used has been modeled after the Robolase system described by Botvinick and Berns, 2005. This system allows for control of imaging and ablation parameters including the region to be targeted, laser power, and length of exposure. By varying the number of pulses in x, y, and z axis at a focal spot irradiance of 4.2x1011 W/cm2, we observe the extent of damage to include or exclude multiple centrosomal components including centrin, pericentrin, and gamma tubulin as determined by immunofluorescence staining. The Robolase software also allows for brightfield and fluorescence images taken immediately prior to and following laser exposure in a single image or by time sequence. Images acquired from the robolase system showed either fragmentation or complete loss of all fluorescently labeled centrosomal components. Live imaging in combination with different laser ablation parameters are being used to study cell migration following the loss of the centrosomal components. Our results show that we can precisely and successfully ablate multiple centrosomal components in directionally migrating cells.

1136/B344

Protein Localization during Basal Body Assembly in Naegleria gruberi.

S. Chun[1], L. Fritz-Laylin[1], C. Fulton[2], Z. Cande[1]; [1]Molecular and Cell Biology, UC Berkeley, Berkeley, CA, [2]Biology, Brandeis University, Waltham, MA

As the organizer of both cilia/flagella and the pericentriolar material required for mitotic spindle formation, centrioles and basal bodies must replicate exactly once per cell cycle. The most commonly studied method of centriole/basal body duplication, termed “templated” assembly, involves new centrioles appearing adjacent to a preexisting centriole. *Naegleria gruberi* is an “amoebo-flagellate”. Normally, *Naegleria* exist as amoeba, dividing using a centriole-free, intranuclear mitotic spindle. Once stressed, the amoeba make basal bodies, flagella, and cytoplasmic microtubules *de novo*, free from preexisting “mother” centrioles and independent from the cell cycle. Over 90% of cells in culture form flagella in a 20-minute window. The resulting flagellates are transient, and will revert to amoeba within several hours. We are currently developing the protist *Naegleria gruberi* as a model system to determine the order and regulation of *de novo* basal body assembly. Centrin is a calcium-binding protein required for the duplication of centrioles. Our high-resolution microscopy studies detail centrin and tubulin localization changes during *Naeglerial* differentiation. Tubulin localizes to basal body precursors along with centrin in earlier time points. Centrin localization initiates at 20 minutes as a single round focus, then at 35 minutes develops into two foci, one bar-shaped and the other round, and finally at 40 minutes localizes in two bar shaped foci, likely representing basal body configuration in the flagellate stage. SAS-6 is a coiled-coil protein found in *Caenorhabditis elegans* that is required for daughter centriole formation, and has a homolog in *Naegleria*. Having both the sequence of localization for both centrin and tubulin, we are initiating studies to determine SAS-6’s position in the *Naegleria* basal body assembly pathway.

1137/B345

Investigating the Importance of Aurora-A Kinase Activity in the Early C. elegans Embryo.

N. Portier[1], F. Motegi[2], M. Bush[1], A. Dammermann[1], A. B. Desai[1], K. Oegema[1]; [1]Ludwig Inst. for Cancer Research, UC San Diego, La Jolla, CA, [2]Molecular Biology and Genetics, Johns Hopkins School of Medicine, Baltimore, MD

Aurora-A is a mitotic serine/threonine kinase that localizes to centrosomes, the major microtubule organizing centers in animal cells. The gene encoding Aurora-A is amplified in many cancers, possibly because overexpression overrides critical checkpoints, promoting genetic instability. However, how Aurora-A overexpression results in cancer has not been fully elucidated. The kinase has been shown to have multiple functions throughout the cell cycle. Aurora-A has been shown to regulate both the recruitment of pericentriolar material during mitotic entry (centrosome maturation), and G2/M progression. Additionally, Aurora-A kinase plays a role in polarity establishment, spindle formation, microtubule nucleation, and timely nuclear envelope breakdown. Previous studies have monitored the consequences of Aurora-A depletion using RNAi and mutants, however the role of the kinase activity specifically has not been investigated. We have developed strains expressing RNAi-resistant wild-type (WT) and kinase-dead (KD) Aurora-A (AIR-1). Depletion of endogenous protein by RNAi leaves the re-encoded WT or KD protein in the early embryos. We have used a variety of methods to monitor AIR-1 function including in vitro kinase assays to confirm loss of Aurora-A activity, fluorescence microscopy to monitor pericentriolar material accumulation, and localization of polarity markers in the absence of Aurora-A phosphorylation. In addition, a quantitative light-microscopy-based live assay to measure chromosome condensation was used to probe whether the interval between the onset of chromosome condensation and NEBD was increased in the absence of Aurora-A kinase activity. Our results shed light on the role of Aurora-A kinase activity in its multiple functions in the C. elegans early embryo.

1138/B346

Loss of Dictyostelium Centrin B Results in Centrosome and Nuclear Abnormalities.

S. Mana-Capelli, D. Larochelle; Biology, Clark University, Worcester, MA

Centrins are calcium-binding proteins related to calmodulins. Diverse roles have been attributed to these proteins in different eukaryotic systems, including MTOC duplication and maintenance, DNA repair, mRNA export, and organelle segregation. DdCenB, one of the two centrins of *Dictyostelium discoideum*, is confined to the cell nucleus during interphase and until the beginning of mitosis. In our effort to characterize the functional role of DdCenB, we have recently developed a DdCenB knock out cell line. DdCenB was targeted by homologous recombination and the DdCenB locus disruption was confirmed by PCR. Cells lacking DdCenB are characterized by nuclear and centrosome-related phenotypes, including by very large nuclei, disruption of the nucleus-centrosome attachment, and multiple centrosome abnormalities. These abnormalities ranged from morphological changes to supernumerary MTOCs. Additionally, immunostaining with different antibodies raised against centrosome components showed smaller “bodies” of different shapes that had limited or no microtubule nucleation capabilities. Finally, mitotic cells displayed aberrant spindles with abnormal centrosome composition (based on cross-reactivity with selected antibodies).

The D. discoideum centrosome cycle is synchronized with mitosis where ingression of the centrosome into the nuclear envelope occurs at the onset of mitosis. Therefore, we propose that before disappearing from the nucleus, DdCenB is part of the machinery that allows for proper centrosome cycle dynamics, including insertion in the nuclear envelope (as in budding yeast), and corona-related protein reorganization. Loss of function of DdCenB consequently leads to improper centrosome division and maintenance.

1139/B347

miR-15a Modulates Cdc25A Protein Expression, Cholangiocyte Proliferation and Centrosome Function: Implications for Hepatic Cystogenesis.

T. Masyuk[1], S. Lee[2], P. Splinter[1], J. Banales[1], A. Masyuk[1], A. Stroope[1], N. LaRusso[1]; [1]Internal Medicine, Mayo Clinic College of Medicine, Rochester, MN, [2]Chonbuk National University Medical School, Jeonju, South Korea

Objective: In Autosomal Recessive Polycystic Kidney Disease (ARPKD), increased cholangiocyte proliferation is involved in hepatic cystogenesis. We previously demonstrated accelerated cholangiocyte proliferation in ARPKD and speculated that cell cycle dysregulation, centrosome amplification, and altered miRNA expression were contributing mechanisms. Thus, our aims were to determine the expression of the cell cycle protein, Cdc25A, and the miRNA profiles in PCK-CCL (a cell line derived from the PCK rat, an animal model of ARPKD). We also assessed the effect of experimental modulation of Cdc25A by an miRNA (i.e., miR15a) on cell proliferation. Methods: Proliferation of normal rat cholangiocytes (NRCs) and PCK-CCL was determined by MTS absorbance. Expression of Cdc25A was assessed by Western blot and RT-PCR. Centrosomal topography was observed by confocal microscopy using gamma tubulin antibody. miRNA profiles were examined by microarray analysis and RT-PCR, and potential miRNA targets identified by in silico screening. Cdc25A level and rate of proliferation were also examined in PCK-CCL transfected with an miRNA precursor. Results: In PCK-CCL, Cdc25A was overexpressed 3-fold compared to NRC. PCK-CCL had a higher percentage (30%) of cells with amplified centrosomes than NRCs (6%). PCK-CCL exhibited global changes in miRNA expression with the majority of them being down-regulated. One of miRNAs, miR-15a, which has complementarity to Cdc25A mRNA, was decreased 37-fold in PCK-CCL compared to NRCs. Compared to non-transfected cells, transfection of the PCK-CCL with a miR-15a precursor: (1) inhibited Cdc25A protein level up to 50% ($p<0.05$); (2) decreased the rate of cell proliferation up to 20% ($p<0.05$); and (3) diminished the percentage of cells with hyper-amplified centrosomes by 10% ($p<0.05$). Conclusions: In the PCK-CCL, abnormal cell proliferation is associated with Cdc25A overexpression, centrosomal hyperamplification and miR15a down-regulation. Experimental up-regulation of miR-15a decreases the rate of proliferation and reduces centrosomal hyperamplification. The data suggest that miR-15a post-transcriptionally modulates Cdc25A resulting in altered cell cycle phenomena contributing to hepatic cystogenesis.

1140/B348

De Novo Basal Body Formation in Naegleria gruberi.

L. Fritz-Laylin[1], S. Prochnik[2], S. Chen[1], D. Rokhsar[1, 2], Z. Cande[1]; [1]UC Berkeley, Berkeley, CA, [2]DOE Joint Genome Institute, Walnut Creek, CA

As the organizer of both cilia/flagella and the pericentriolar material required for mitotic spindle formation, centrioles and basal bodies must replicate exactly once per cell cycle. The most commonly studied method of duplication, termed "templated" assembly, involves new centrioles appearing adjacent to a preexisting centriole. *Naegleria gruberi* is an "amoebo-flagellate". Normally, *Naegleria* exist as amoeba, dividing using a centriole-free, intranuclear mitotic spindle. Once stressed, the amoeba make basal bodies, flagella, and cytoplasmic microtubules *de novo*, free from preexisting "mother" centrioles and independent from the cell cycle. Over 90% of cells in culture form flagella in a 20-minute window. The resulting flagellates are transient, and will revert to amoeba within several hours. We are currently developing *Naegleria gruberi* as a model system to determine the order and regulation of *de novo* basal body assembly, using comparative genomics, proteomics and microarrays. These three approaches have identified 38 putative *Naegleria* basal body components, including several novel proteins. An initial map of *de novo* basal body formation in *Naegleria* was made by immunolocalization of *Naegleria* centrin, and alpha, beta and gamma tubulins. We are currently developing antibodies to additional components to further describe *Naegleria*'s *de novo* basal body assembly process.

1141/B349

The Control of Centrosome Disengagement and Duplication.

W. Wang[1], K. A. Yule[2], P. V. Jallepalli[2], T. Stearns[3], M. Tsou[1, 3]; [1]Cell Biology, MSKCC, New York, NY, [2]Molecular Biology, MSKCC, New York, NY, [3]Biological Sciences, Stanford University, Stanford, CA

The centrosome duplicates only once per cell cycle in dividing cells. A simple way to achieve this control is to temporally separate the activity that licenses the duplication from the assembly activity that physically builds the centriole. The key feature of this model is that the license is not part of the assembly activity, but without it, centriole assembly is not permitted. Here we described such a mechanism in human cells. We showed that separase and polo-like kinase 1 (Plk1) collaborate in mitosis to disengage centrioles, a process liberates the assembly of new centrioles in the subsequent interphase. Importantly, neither separase nor Plk1 is required in S phase for centriole assembly, demonstrating for the first time that centriole duplication can be blocked by inactivating molecules that are not part of S-phase activities or components for centriole assembly. From these results, we conclude that (1) there is indeed a licensing process for centriole duplication, (2) this process occurs in mitosis through centriole disengagement, and (3) it requires separase and Plk1. We currently seek to identify the substrate for this licensing activity, and determine if separase and Plk1 are involved in abnormal centrosome amplification often seen in cancer.

1142/B350

Mps1 Phosphorylates Centrin2 to Regulate Centriole Duplication.

C. Yang, C. Kasbek, H. Fisk; Molecular Genetics, The Ohio State University, Columbus, OH

The centrosome is a single copy microtubule-organizing center consisting of a pair of centrioles surrounded by a pericentriolar matrix. Because centrosomes determine the polarity of the mitotic spindle and control chromosome segregation, the single centrosome inherited at cytokinesis must be duplicated prior to mitosis. Errors in centrosome duplication such as the production of excess centrioles within a single cell cycle, lead to the production of abnormal spindles that cause chromosomes to distribute improperly. This produces aneuploid cells such as found in tumors, and extra centrosomes are observed in a variety of aneuploid human tumors. Mps1 is a protein kinase that regulates centrosome duplication; increasing Mps1 dosage results in centriole overproduction, whereas the depletion of Mps1 kinase activity causes the failure of centrosome duplication. It is therefore important to know what the substrates of Mps1 are in order to determine the precise function of Mps1 in centrosome duplication. Centrins are small calcium binding EF-hand proteins that localize to centrosomes. We identified Centrin2 as an Mps1 substrate. Using mass spectrometry, we have identified three sites within Centrin2 that are phosphorylated by Mps1. Mutating T118 within Centrin2 to alanine significantly reduced its phosphorylation by Mps1, suggesting that in vitro T118 is the major Mps1 phosphorylation site. Examination of HeLa cells overexpressing GFP-Centrin2 by fluorescence and electron microscopy after a prolonged S phase revealed an abnormal number and/or arrangement of centrioles that was largely reversed by depletion of Mps1. The production of extra centrioles was attenuated by the non-phosphorylatable T118A mutation, and GFP-Cetrin2T118A is not efficiently incorporated into centrioles. In contrast, the production of extra centrioles was greatly enhanced by the phosphomimetic T118D mutation. These findings suggest that Mps1 regulates centriole duplication by modulating incorporation of Centrin2 into centrioles via phosphorylation at T118.

1143/B351

Antizyme Inhibits Centrosome Reduplication by Restraining the Accumulation of Mps1 at Centrosomes.

C. Kasbek, C. Yang, H. Fisk; the Ohio State University, Columbus, OH

Centrosome reduplication, characterized by multiple rounds of duplication within a single cell cycle, is a mechanism that is thought to lead to aneuploidy. Extra centrosomes are present in many cancers and are especially prevalent in breast cancer. Mps1, a dual specificity kinase, is required for centrosome duplication and misregulation of Mps1 can lead to centrosome defects. Mps1 is regulated at the level of protein stability; phosphorylation by cyclinA/cdk2 at threonine 468 (T468) blocks proteasome-mediated degradation and allows Mps1 to accumulate at centrosomes. This regulation is centrosome-specific in S-phase, but the factors and mechanisms targeting Mps1 for degradation have not been characterized, and we have been unable to document Mps1 ubiqutination. Mangold et al. (Oncogene 2008) demonstrated that Antizyme (OAZ1, a known mediator of ubiquitin-independent degradation) and its antagonist Antizyme Inhibitor (AZI) localize to centrosomes, and that either depleting OAZ1 or increasing AZI causes centrosome reduplication. We have found that this reduplication is abrogated by Mps1 siRNA, suggesting that OAZ1 regulates centrosome number by controlling Mps1 degradation. We have also found that Mps1 and OAZ1 are present in a complex, and that the depletion of OAZ1 leads to an increased level of Mps1 at centrosomes. Moreover, increasing OAZ1 levels by AZI siRNA decreases Mps1 centrosomal levels and abrogates centrosome reduplication in U2OS cells. To test whether OAZ1 is part of the cdk2-regulated Mps1 degradation pathway, we are examining the effect of depleting OAZ1 or AZI on the accumulation of Mps1 degradation signal mutants at centrosomes. Together, our data suggests that Antizyme inhibits centrosome duplication through the cdk2-regulated Mps1 degradation pathway by removing non-phosphorylated forms of Mps1 from centrosomes.

1144/B352

SAK/PLK4 and the Control of Centrosome Number.

I. Cunha-Ferreira[1,2], A. Rodrigues-Martins[1,2], I. Bento[1], D. Glover[2], M. C. Bettencourt-Dias[1]; [1]Instituto Gulbenkian de Ciencia, Oeiras, Portugal, [2]University of Cambridge, Cambridge, United Kingdom

The centrosome is the primary microtubule-organizing centre in animal cells facilitating the organization of the spindle poles during mitosis. Abnormalities in centrosomes occur in many cancers and can be associated with genomic instability. In addition, the core centrosomal components, the centrioles, have another quite distinct function as basal bodies that seed the growth of cilia and flagella, which have critical roles in physiology, development and disease. Therefore, in the healthy organism, strict control of centrosome and centriole number is imperative. Duplication of the centrosome is triggered and dependent on the activity of a conserved kinase, SAK (also known as PLK4). Here we show that centrosome amplification is inhibited by degradation of SAK. We demonstrate that SAK is targeted for degradation by the SCF/Slimb ubiquitin ligase. This complex physically interacts with SAK, and in its absence, SAK levels accumulate. This interaction is mediated via a conserved Slimb binding motif in SAK. Mutations in this degron impair the interaction with Slimb, stabilise SAK levels and lead to centrosome amplification. Since the SCF/Slimb complex, also known as SCF/b-Trcp in mammals, plays an important role in cell cycle progression and DNA damage checkpoints, these results lead to new understanding on the control of centrosome number and how it may go awry in human disease.

1145/B353

Characterisation of Two Novel Drosophila Proteins Implicated in Centriole Duplication: DCP110 and DCep135.

A. Franz, J. Dobbelaere, J. Raff; Gurdon Institute, Cambridge, United Kingdom

In animal cells the centrosome must be duplicated once per cell cycle. Genome-wide studies in the early *C. elegans* embryo only identified five centriole-associated proteins required for centrosome duplication: ZYG-1, SAS-4, SAS-5, SAS-6 and SPD-2. Several of these proteins appear to comprise a conserved duplication pathway that functions in organisms like *Drosophila*. Emerging evidence suggests that other factors are also required for this process. To gain better knowledge of how centriole duplication works on the molecular level, we recently performed a genome-wide RNAi screen in *Drosophila* S2R+ cells, which identified several novel proteins required for centriole duplication, including the uncharacterised proteins encoded by CG14617 and CG17081. The human homologues of these coiled-coil proteins, CP110 and Cep135, localise to centrioles and are required for the Plk4-induced centriole overduplication in human cells. This suggests that they are essential for centriole duplication. We are currently characterising the function of CG14617 (DCP110) and CG17081 (DCep135). We have generated antibodies and GFP-fusions of both proteins and shown that they localise to centrioles in syncytial embryos, primary spermatocytes and larval neuroblasts. In addition we have generated mutants and are currently analysing their phenotypes. *DCP110* mutant flies are viable and fertile, and have no major centriole duplication defects. Interestingly, preliminary results indicate that centrioles in *DCP110* mutant

primary spermatocytes are longer than in the wt. This suggests that, in *Drosophila*, DCP110 is not essential for centriole replication but might regulate centriole length.

1146/B354

Centrosome Amplification Can Initiate Tumorigenesis in Flies.

R. Basto[1], J. Raff[2]; [1]UMR144, Institut Curie, Paris, France, [2]Wellcome Gurdon Institute, Tennis Court Rd, Cambridge, United Kingdom

Centrosome amplification is a common feature of many cancer cells, and it has been previously proposed that centrosome amplification can drive genetic instability and so tumorigenesis. To test this hypothesis, we generated Drosophila lines that have extra centrosomes in □60% of their somatic cells. Many cells with extra centrosomes initially form multipolar spindles, but these spindles ultimately become bipolar. This requires a delay in mitosis that is mediated by the spindle assembly checkpoint (SAC). As a result of this delay, there is no dramatic increase in genetic instability in flies with extra centrosomes, and these flies maintain a stable diploid genome over many generations. The asymmetric division of the larval neural stem cells, however, is compromised in the presence of extra centrosomes, and larval brain cells with extra centrosomes can generate metastatic tumors when transplanted into the abdomens of wild-type hosts. Thus, centrosome amplification can initiate tumorigenesis in flies.

1147/B355

Loss of Centriole Engagement and Cohesion Results in Multiple Centrioles and Primary Cilia in CDK5RAP2 Mutant Mice.

J. Barrera[1,3], L. R. Kao[1,3], R. E. Hammer[2,3], T. Megraw[1,3]; [1]Pharmacology, UT Southwestern Medical Center, Dallas, TX, [2]Biochemistry, UT Southwestern Medical Center, Dallas, TX, [3]Green Center for Reproductive Biology, UT Southwestern Medical Center, Dallas, TX

Autosomal recessive primary microcephaly (MCPH) is a rare developmental disorder resulting in reduced brain size. Mutations in three of the four genes linked to MCPH affect centrosomal proteins, implicating a centrosomal requirement in brain development. One of these genes, Cyclin-dependent Kinase 5 Regulatory Associated Protein 2 (CDK5RAP2) localizes to centrosomes and is an apparent homolog of Drosophila Centrosomin. We derived two CDK5RAP2 mutant mice that closely mimic the two mapped human truncations. The involvement of CDK5RAP2 in centrosome structure and regulation was investigated in mouse embryonic fibroblasts (MEFs). CDK5RAP2 mutant MEFs showed centriole amplification. Moreover, the usual paired configuration of centrioles in centrosomes was lost. Centrosomes within CDK5RAP2 mutant MEFs vary in their centriole composition, being comprised of single, paired or triplet centrioles. Most mammalian cells contain a single primary cilium, essential for intercellular signaling, templated from the "mother" centriole. While the incidence of cilium formation was unaffected, multiple centrioles resulted in the formation of multiple mature mother centrioles that contributed to the formation of multiple primary cilia in 33.3% of CDK5RAP2 mutant MEFs, compared to 4.7% in wild-type MEFs. Co-immunoprecipitation assays identified pericentrin as an interacting partner of CDK5RAP2. Localization of pericentrin was unaffected, but localization of rootletin was altered in CDK5RAP2 mutant MEFs. In wild-type MEFs, CDK5RAP2 and rootletin co-localized to fibrous non-microtubule structures that emanate from centrosomes, whereas in CDK5RAP2 mutant MEFs rootletin localization was restricted to centrosomes. Current models predict that loss of centriole engagement initiates centriole licensing, thereby activating extra rounds of centriole duplication. Our data indicate that CDK5RAP2 is required to maintain centriole engagement, in addition to the cohesion that maintains mother centriole proximity until mitotic onset. Thus, CDK5RAP2 restricts centriole duplication. The impact of this disruption on mouse physiology is under investigation.

1148/B356

A Role for Beta-Catenin and Nek2 in Extra Centrosome Assembly.

A. Barth[1,2], S. Bahmanyar[1,2], E. L. Guiney[2], W. Nelson[1,2]; [1]Biological Sciences, Stanford University, Palo Alto, CA, [2]Molecular and Cellular Physiology, Stanford University School of Medicine, Palo Alto, CA

Centrosomes have a dominant role in spindle assembly in mitosis, and produce multipolar spindles when present in abnormal numbers. Despite the importance of centrosomes in cell division, the assembly of extra centrosomes, a hallmark of many cancers, is poorly understood. The oncogene beta-catenin, known for its role in cell adhesion and Wnt signaling, has been shown to function in centrosome separation as a substrate for the kinase Nek2. Increased levels of beta-catenin and Nek2 are common to many types of cancers, and deregulation of Nek2 activity causes centrosome amplification. We found that expression of mutant beta-catenin results in multipolar spindles. Interphase cells expressing mutant beta-catenin have functional and non-functional centrosomal structures. Non-functional structures contain gamma-tubulin and centrin but not pericentrin, and have increased amounts of PCM-1, a centriolar satellite protein involved in delivery and assembly of centrosomal components. Live cell imaging of PCM-1 and centrin revealed an immobile population of PCM-1 satellites that were associated with centrin aggregates and resembled de novo centrosomes. Overexpression of Nek2 in wild type cells increases phosphorylation of PCM-1 and decreases centrin levels suggesting that Nek2 regulates PCM-1 and centrin. Recruitment of Nek2 to centrosomes in mutant beta-catenin cells was normal and we are currently testing whether Nek2 assembly or activity is perturbed. Taken together, these results suggest a pathway for extra centrosome assembly, and provide a potential mechanism by which mutant beta-catenin promotes cancer progression.

1149/B357

Bardet-Biedl Syndrome Proteins Play a Role in Microtubule Nucleation and Centrosome Assembly.

D. L. Kooyman[1], J. Wei[2,3], V. C. Sheffield[2,3]; [1]Physiology & Developmental Biology, Brigham Young University, Provo, UT, [2]Department of Pediatrics, University of Iowa, Iowa City, IA, [3]Howard Hughes Medical Institute, Iowa City, IA

The centrosome is a functionally complex organelle essential for both mitotic spindle and primary cilium formation in its role as a center for microtubule nucleation and organization. It appears to be involved with numerous other cellular functions as well. Bardet-Biedl syndrome (BBS) is a pleiotropic genetically heterogeneous disorder characterized by obesity, retinopathy, polydactyly, renal malformations, learning disabilities, and hypogenitalism. Although the cellular functions of BBS proteins are not yet fully understood, evidence from a variety of organisms, including BBS model mice, suggests that BBS proteins play a role in cilia assembly and/or function, as well as intracellular vesicle trafficking. The purpose of this study was to identify interactions of BBS proteins with centrosome assembly and microtubule

nucleation. Using BBS knockout mice as model organisms we report that Bbs1 and Bbs2 associate with the centrosome and have a role in its assembly and regulation. The capacity of the centrosome to act as a center for nucleation of microtubules was attenuated in the Bbs1 M390R missense mutant or in the absence of Bbs2 and Bbs4. Bbs1 was also shown to regulate the level and stability of gamma-tubulin, which, in turn, is necessary for regulation of centrosome-dependent and centrosome-independent microtubule dynamics during mitotic spindle formation.

Kinetochores I (1150 – 1171)

1150/B359

Quantitative Characterization of CENH3 Chromatin in Megabase-scale Arabidopsis Centromeres.

R. Maruthachalam, J. Ramahi, P. Kwong, J. Stewart, R. Menorca, S. Chan; Plant Biology, UC Davis, Davis, CA

Centromeres are essential for chromosome segregation, yet their DNA sequences and composition evolve rapidly. Most plants and animals have "regional centromeres", comprising megabase scale tandem repeat arrays. An unknown fraction of the array is bound by nucleosomes containing the centromere-specific histone H3 variant CENH3 (CENP-A in humans). S. cerevisiae centromeres contain only one CENH3/Cse4p nucleosome, and the stoichiometry of their kinetochore proteins has been described. We are using the model plant Arabidopsis thaliana to ask 1) How many CENH3 nucleosomes are found in a large regional centromere? 2) What sequences are required for CENH3 targeting to regional centromeres? A cenh3 null mutation in Arabidopsis (htr12-1 or cenh3-1) is embryo lethal, but can be fully complemented by GFP-tagged CENH3 expressed from the endogenous promoter. A range of complementation was observed in transgenic GFP-CENH3 lines, providing tools for future investigation of the minimum functional size of kinetochores in a regional centromere. We used S. cerevisiae Cse4-GFP and Ndc80-GFP as fluorescence microscopy standards to count the number of GFP-CENH3 molecules in single plant kinetochores. Arabidopsis metaphase kinetochores contained approximately 90 CENH3 nucleosomes. This may underestimate the wild type value, as the cenh3-1 GFP-CENH3/+ parent plant from the imaged line had higher than expected embryo lethality, showing lack of full complementation. We have also expressed several CENH3/H3 chimeras in Arabidopsis as GFP fusions. The CENH3 histone fold domain is necessary and sufficient for kinetochore localization, but the CENP-A targeting domain (CATD) is not sufficient (unlike in human cells). A CENH3 histone fold domain containing the N-terminal tail of conventional H3.3 complements cenh3-1, allowing us to manipulate centromere histone modifications in the absence of endogenous CENH3. We have shown that Arabidopsis genetics facilitates quantitative analysis of chromatin in regional centromeres.

1151/B360

Analysis of CENP-A Replenishment in Early G1.

J. F. Dorn, A. Lagana, P. S. Maddox; Institute for Research in Immunology and Cancer, University of Montreal, Montreal, QC, Canada

Inhibiting cell division remains one of the most important anti-cancer strategies. However, the two most prominent drug targets - microtubules and kinases - perform important functions outside of mitosis. To reduce the side-effects of anti-cancer drugs, it is therefore necessary to identify proteins that are exclusively required for mitosis, and whose disruption critically disrupts mitosis. The histone H3 variant CENP-A that functions as the epigenetic mark for centromere localization fulfills all these criteria. Targeted inhibition of CENP-A localization to the centromere requires detailed understanding of its dynamics. Recent research has demonstrated that CENP-A, which is distributed between the daughter centromeres during replication, is replenished to mother levels through incorporation of newly synthesized protein only after mitotic exit. However little is known about the exact mechanisms and the proteins involved in either process. Here, we present an analysis of CENP-A loading in early G1. We measure CENP-A content at individual centromeres by high-resolution 4D microscopy and tracking of fluorescently labeled CENP-A in HeLa cells. We find that CENP-A gets incorporated into chromatin at an average rate of ~5 molecules per minute per centromere, and that it takes ~6 hours for CENP-A intensity to return to pre-duplication levels. Thus, the speed of CENP-A incorporation is on the order of magnitude of the speed of histone deposition following transcription, and CENP-A replenishment is complete well before the onset of S phase. The exponential replenishment dynamics are compatible with a model allowing the exchange of centromeric and soluble CENP-A, and centromeric CENP-A seems to be stabilized by HsKNL2^{M18BP1} and the KNAP (KNL2 associated protein) proteins.

1152/B361

HsKNL-2 M18BP1 Associated Proteins Implicated in Centromere Function.

A. Lagana, A. Lavoie-Lennon, J. F. Dorn, V. De Rop, P. S. Maddox; Institute for Research in Immunology and Cancer, University of Montreal, Montreal, QC, Canada

Centromere integrity is crucial for kinetochore structure, assembly and association of the chromosomes with the microtubules during cell division. HsKNL-2^{M18BP1} is a MYB domain DNA binding protein required for CENP-A localization to the centromere and chromosome segregation. However the mechanism of HsKNL-2^{M18BP1} in CENP-A loading is as yet unknown. To better understand HsKNL-2^{M18BP1} function and mechanisms we co-immunoprecipitated HsKNL-2^{M18BP1} associated proteins from Microccocal Nuclease treated Hela cell lysates with two different specific antibodies directed against HsKNL-2^{M18BP1}. Proteomic analysis and comparison to two non-specific control immunoprecipitations identified a small subset of proteins specifically associated with HsKNL-2 ^{M18BP1}. Among the specific proteins were HsKNL-2^{M18BP1} itself as well as Mis-18, RbAp46, and RbAp48, a protein complex which has previously been identified by similar methods. 4 additional unique proteins were present in the HsKNL-2^{M18BP1} sample which we named KNAP (KNL-2 Associated Protein) 1-4. GFP fusion to KNAP1 and KNAP2 show partial colocalization with centromeres during G1 of the cell cycle, the time when HsKNL-2^{M18BP1} also localizes to the centromere. We hypothesize that the partial association of KNAP1 and KNAP2 with CENP-A might indicate a dynamic transient association of these proteins with the centromere. KNAP1 depletion by short hairpin transfection causes a partial (~60%) loss of CENP-A localization at the centromere. We will present data which probes the role of these proteins in CENP-A loading, kinetochore function and chromosome segregation.

1153/B362

Investigating the Role of Histone H4 Acetylation in Centromeric Chromatin Assembly.

K. Godek, C. Carroll, A. Straight; Biochemistry, Stanford, Stanford, CA

Accurate chromosome segregation is essential for proper cell division. Each eukaryotic chromosome possesses a specialized region of chromatin called the centromere that is necessary for chromosome attachment to the mitotic spindle and chromosome segregation. Centromere protein A (CENP-A) is a histone H3 variant that replaces histone H3 in centromeric nucleosomes and is required for centromere function. It is not known how CENP-A nucleosomes are assembled exclusively at centromeres. To identify factors that might participate in CENP-A nucleosome assembly, we performed affinity chromatography experiments with the N-terminal tail of CENP-A. We identified that histone acetyltransferase1 (Hat1) and retinoblastoma associated proteins 46/48 (RbAp46/48) bind to the CENP-A N-terminal tail. We have shown that the Hat1/RbAp46 complex acetylates histone H4 in both H3/H4 and CENP-A/H4 tetramers. We are currently testing whether acetylation mimics and non-acetylatable versions of histone H4 affect the assembly of centromeric chromatin.

1154/B363

Analysis of Xenopus Centromere Assembly.

B. Moree, A. Straight; Biochemistry, Stanford Medical School, Stanford, CA

The eukaryotic kinetochore acts as the interaction site for chromosomes with the mitotic spindle and ensures proper chromosome alignment and segregation during mitosis. Kinetochores are assembled only in mitosis but the site of kinetochore assembly, the centromere, persists throughout the cell cycle. Centromeric chromatin is unique in that histone H3 is replaced in nucleosomes by the essential centromeric histone variant, centromere protein A (CENP-A). CENP-A chromatin serves as a platform for at least 15 other centromere specific factors that are required for mitotic kinetochore assembly and chromosome segregation. In order to study centromere assembly we have used meiotic *Xenopus laevis* egg extracts as an in vitro system for centromere protein assembly onto *Xenopus* sperm chromatin. We have isolated genes for the *Xenopus* centromere proteins from the Nucleosome Associated Complex (NAC), CENP-A Distal Complex (CAD), and Mis12 Complex and verified their localization in *Xenopus* somatic cells. We have analyzed the assembly of these proteins onto *Xenopus* sperm centomeres by translating epitope tagged centromere proteins in *Xenopus* egg extracts and by immunolocalization of the endogenous proteins. In contrast to our observations in somatic cells, we find that only a subset of centromere proteins are able to associate with centromeres in *Xenopus* extract. The essential centromere proteins CENP-H and CENP-I are not detectable at centromeres in egg extracts while CENP-C and CENP-A localize normally. These results suggest that the mitotic and meiotic centromeres may have distinct organizations that give rise to functional differences in mitosis and meiosis.

1155/B364

Analysis of the Role of CENP-C in Centromere Assembly.

K. J. Milks, B. Moree, A. Straight; Department of Biochemistry, Stanford University School of Medicine, Stanford, CA

Kinetochores attach chromosomes to the mitotic spindle, monitor proper chromosome alignment, and couple chromosomes to spindle forces during cell division. The centromere is a specialized region on each chromosome that serves as the assembly site for the mitotic kinetochore. During interphase, centromeres are constitutively bound by >15 centromere-specific proteins that are essential for mitotic kinetochore assembly. How the centromere is assembled and how it promotes mitotic kinetochore formation are unknown. We have used *Xenopus laevis* egg extract as an *in vitro* centromere assembly system to analyze the centromere assembly process. Centromeric chromatin is characterized by the presence of the essential histone H3 variant, centromere protein A (CENP-A). CENP-A chromatin directs the assembly of most other centromere proteins. An obligate step in centromere assembly is the localization of another centromere protein, CENP-C, specifically to CENP-A-containing chromatin. CENP-A persists in sperm chromatin through meiosis to mark the site of centromere assembly at fertilization. We show that CENP-C is not maintained in sperm chromatin through meiosis but is assembled from the egg cytoplasm when sperm is added to egg extract. Immunodepletion of CENP-C from egg extract prevents centromere and kinetochore formation, and depleted extracts can be complemented with *in vitro* translated CENP-C. We have generated a series of CENP-C mutants and analyzed the ability of these mutants to target to CENP-A chromatin and to support centromere and kinetochore assembly. CENP-C amino-terminal truncations target to the centromere normally but do not support kinetochore formation. We are currently determining which centromere proteins are targeted to centromeres through the amino terminus of CENP-C to understand how CENP-C promotes centromere and kinetochore formation.

1156/B365

Centromere Recognition and Assembly.

C. Carroll, K. Godek, A. Straight; Biochemistry, Stanford School of Medicine, Palo Alto, CA

Centromeres are specialized chromatin domains that direct kinetochore assembly during mitosis. How centromeric chromatin is recognized as the site of kinetochore assembly is not understood. A key component of functional centromeres in all eukaryotes is the histone H3 variant CENP-A. CENP-A replaces histone H3 in centromeric nucleosomes and is required for kinetochore assembly. We have reconstituted nucleosomes that contain either histone H3 or CENP-A and used these nucleosomes to identify proteins that are important for centromere recognition. CENP-C and CENP-N, two constitutive centromere proteins, bind directly and specifically to CENP-A-containing nucleosomes. Nucleosomes reconstituted with a chimeric histone in which the CENP-A targeting domain (CATD) of CENP-A replaced the corresponding region of histone H3 were sufficient for binding to CENP-N but not CENP-C, indicating that CENP-N and CENP-C recognize distinct structural elements of centromeric nucleosomes. Mutations that disrupt CENP-N nucleosome binding in vitro affect CENP-N and CENP-K, but not CENP-C, localization in vivo. CENP-A-nucleosome binding by CENP-N is therefore important for some aspects of centromere/kinetochore assembly, but alternative modes of centromere recognition exist in vivo. Depletion of CENP-N with siRNA's resulted in centromere/kinetochore assembly defects that were similar to those displayed by cells expressing CENP-N mutants that are deficient in CENP-A nucleosome binding. These data suggest that CENP-N and CENP-C recognize the chromosomal site of kinetochore assembly via direct and specific interactions with CENP-A nucleosomes.

1157/B366

Recruitment of CenH3, CENP-C and Cal1 to Fly Centromeres.

B. Mellone[1,5,2], S. Erhardt[1,5,4], C. Betts[3], W. Zhang[1,5], V. Shteyn[1,5], I. Oderberg[1,5], A. Straight[3], G. Karpen[1,5]; [1]Department of Genome and Computational Biology, Lawrence Berkeley National Laboratory, Berkeley, CA, [2]Molecular and Cell Biology Department, University of Connecticut, Storrs, CT, [3]Biochemistry, Stanford University, Stanford, CA, [4]ZMBH, Universität Heidelberg, Heidelberg, Germany, [5]Molecular and Cell Biology, University of California Berkeley, Berkeley, CA

Normal inheritance of genetic material requires that chromosomes segregate faithfully during mitosis and meiosis. The centromere is a specialized chromosomal site that is the structural and functional foundation for kinetochore formation. Centromeres are regulated epigenetically, and must be reconstituted after each replication cycle to prevent progressive dilution of the epigenetic mark, characterized by the presence of the centromere specific histone CenH3. Using genome-wide RNAi screening for defects in CenH3 localization in Drosophila, we identified the centromere proteins CAL1 and centromere protein C (CENP-C) as essential factors for CenH3 localization at the centromere. CenH3, CAL1 and CENP-C co-immunoprecipitate, are mutually dependent for centromere localization, and function by affecting both recruitment and maintenance of CenH3 at centromeres. Others have reported that, unlike canonical histone H3.1, which is recruited during S phase, CenH3 is recruited in late mitosis/G1 in human cells and early drosophila embryos. However, early drosophila embryos lack G1 and G2 phases. To better dissect the cell-cycle dynamics of CenH3 localization in flies, we have used SNAP-tagging and FRAP in S2 cells, which display normal G1 and G2 phases, and evaluated CenH3 recruitment at different cell-cycle stages. We also analyzed the cell-cycle dynamics of CENP-C and Cal1. We will present our current understanding of the interdependent recruitment of CenH3, CENP-C and Cal1 to centromeres.

1158/B367

Architecture and Flexibility of the Yeast Ndc80 Kinetochore Complex.

H. Wang[1], S. Long[2], C. Ciferri[2], S. Westermann[3], D. Drubin[2], G. Barnes[2], E. Nogales[1,2,4]; [1]Life Sciences Division, Lawrence Berkeley National Lab, Berkeley, CA, [2]Molecular and Cell Biology, UC-Berkeley, Berkeley, CA, [3]Research Institute of Molecular Pathology, Vienna, Austria, [4]HHMI, Berkeley, CA

Kinetochores mediate microtubule-chromosome attachment and ensure accurate segregation of sister chromatids. The highly conserved Ndc80 kinetochore complex makes direct contacts with the microtubule and is a likely component essential in spindle check-point signaling. It contains a long coiled-coil region with globular domains at each end involved in kinetochore localization and microtubule binding, respectively. We have directly visualized the architecture of the yeast Ndc80 complex and found a dramatic kink within the 560 Å coiled-coil rod located about 160 Å from the larger globular head. Comparison of our EM images to the structure of the human Ndc80 complex allowed us to position the kink proximal to the microtubule-binding end and to define the conformational range of the complex. The position of the kink coincides with a coil-coiled breaking region conserved across eukaryotes. We hypothesize that the kink in Ndc80 is essential for correct kinetochore geometry and could be part of a tension sensing mechanisms at the kinetochore.

1159/B368

Assembly and Function of the Eukaryotic Kinetochore.

J. P. Welburn, I. Cheeseman; Whitehead Institute for Biomedical Research, Cambridge, MA

The assembly and structure of the kinetochore is highly regulated during the cell cycle and in response to the spindle checkpoint to allow timely and correct chromosome segregation to opposite poles in mitosis. Although great progress has been made to understand the molecular organization of the human kinetochore and those components essential for chromosome division, the regulation and dynamics of this supramolecular assembly remains unknown. In particular, phosphorylation of some of the outer kinetochore proteins by Aurora B has been shown to regulate the microtubule interactions but the effects of kinetochore phosphorylation remain largely unkown. We have identified several phosphosites in vivo by mass spectrometry, some of which we have identified to be phosphorylated by Aurora B and the CDK family of protein kinases sites. We are currently studying the role of CDK and Aurora B phosphorylation on the assembly of kinetochore complexes and on the regulation of the microtubule-kinetochore attachment, using biophysical and biochemical approaches that are combined with cell biology in human cell lines. This hybrid approach enables us to understand the structural and cellular components that underlie the regulation of the kinetochore.

1160/B369

A Map of In Vivo Protein Localization within the Budding Yeast Kinetochore at 10 Nm Resolution.

A. Joglekar, K. Bloom, T. Salmon; U. of N. Carolina, Chapel Hill, NC

The kinetochore is a macromolecular protein machine that links centromeric chromatin to the plus-ends of one or more microtubules and segregates chromosomes during cell division. The core structure of the budding yeast kinetochore consists of eight protein complexes, most of which are conserved in all eukaryotes. Transformation of this protein parts-list into a working assembly is critical for understanding the molecular mechanisms that drive kinetochore function and regulation. We have developed a two-color, in vivo fluorescence microscopy technique to determine the location of budding yeast kinetochore proteins along the kinetochore axis at 10 nm resolution. Using this technique we find that the kinetochore, from the centromeric nucleosome to the microtubule-binding Ndc80 complex, covers 80 nm. The Ndc80 complex spans ~ 54-57 nm with its axis positioned almost parallel to the microtubule axis. It is linked to the centromere through an elongated Mtw1 complex that is at least 15 nm long. Members of the Ctf19 complex are within 5 nm of the N-terminus of Cse4p, which demarcates the centromere. Interestingly, the Dam1-DASH complex localizes 10 nm inside the attachment point between the Ndc80 complex and the microtubule. In anaphase, the length of the kinetochore reduces to ~ 59 nm demonstrating cell-cycle or tension-dependent reorganization. Length of the Ndc80 complex decreases non-uniformly - the Ndc80p/Nuf2p dimer reduces in length more than the Spc24p/Spc25p dimer, suggesting an intra-molecular conformational change. These protein localizations provide a framework for integrating structural information for individual protein complexes. When combined with the copy number per kinetochore for each complex, they enable us to envision the 3-D protein architecture of a kinetochore-microtubule attachment. The in vivo imaging technique developed for the

kinetochore can be employed for integrating protein parts-lists assembled for a wide range of cellular machinery into working mechanisms. [Supported by NIH GM24364 & GM32238].

1161/B370

Protein Architecture of the Human Kinetochore Measured by KSHREC.

X. Wan[1], R. P. O'Quinn[1], H. L. Pierce[1], J. Polka[1], J. G. DeLuca[2], A. Desai[3], S. Liu[4], T. J. Yen[4], A. F. Straight[5], P. T. Stukenberg[6], S. S. Taylor[7], E. D. Salmon[1]; [1]Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, [3]Cellular and Molecular Medicine, Ludwig Institute for Cancer Research, La Jolla, CA, [4]Fox Chase Cancer Center, Philadelphia, PA, [5]Biochemistry, Stanford University, Stanford, CA, [6]School of Medicine, University of Virginia, Charlottesville, VA, [7]Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom

A major goal for understanding how vertebrate kinetochores are assembled and function in mitotic chromosome segregation is knowledge of the protein architecture linking centromeric DNA to the plus ends of kinetochore microtubules (kMTs). Kinetochore speckle high resolution co-localization (KSHREC) is a two-color fluorescence light microscopy method we have developed that has 5-10 nm accuracy for average protein epitope localization along the inner-outer axis through human metaphase kinetochores. Our data provide a new high resolution picture of where key core protein structural elements of the kinetochore are located and oriented relative to one another during metaphase for both control and taxol treated cells. The data also contains mechanical properties of the different protein linkages. Together, the high resolution map and mechanical information: clarify the structural mechanics of the inner kinetochore; demonstrate significant compliance of CENP-A and CENP-C relative to the Ndc80 complex during centromere stretch; indicate a stiff linkage from CENP-I and CENP-T to the Ndc80 complex and other members of the KMN network; support a new model for how the Ndc80, Mis12 and Knl1/Blinkin complexes provide kMT attachment and generate pulling forces from kMT depolymerization; provide evidence for a low tension switch of ~12 nm in the axial position of the Ndc80 complex relative to the Knl1/Blinkin complex; reveal an extraordinary change in the conformation of the MT motor CENP-E; and show the location of the plus ends of kMTs. This research is supported by NIGMS 24364 and 60678.

1162/B371

Meiotic Kinetochore Assembly and Function in the Holocentric Nematode C. elegans.

J. Dumont, K. Oegema, A. B. Desai; Department of Cellular and Molecular Medicine, University of California San Diego - Ludwig Institute for Cancer Research, La Jolla, CA

Kinetochores play an essential role in genome transmission by forming the interface between chromosomes and spindle microtubules. Two types of chromosome architectures are prevalent among eukaryotes: monocentric, in which localized centromeres restrict assembly of kinetochores to focused chromosomal sites, and holocentric, in which diffuse kinetochores assemble along the length of chromosomes. Holocentricity is widely prevalent in metazoans and is observed in distinct phyla such as lower plants, nematodes, and hemipteran insects. We have been investigating kinetochore assembly and function in the holocentric nematode C. elegans. During mitosis, when replicated sister chromatids must be segregated to maintain euploidy, centromeric chromatin directs assembly of conserved outer kinetochore complexes that form attachments to spindle microtubules, in a manner analogous to eukaryotes with monocentric chromosomes. By contrast, during meiosis, when homologous chromosomes must be segregated to reduce ploidy outer kinetochore assembly is uncoupled from centromeric chromatin - instead cup-shaped structures comprised of outer kinetochore proteins form on the surfaces of the bivalent chromosomes immediately prior to nuclear envelope breakdown of meiosis I. To investigate the role of these cup structures, which represent the meiotic kinetochore in C. elegans, we used RNAi-mediated depletion of the conserved outer kinetochore scaffold protein KNL-1. Meiotic kinetochore assembly requires KNL-1 and its depletion leads to chromosome missegregation. Specifically, KNL-1 depleted embryos exhibit a defect in chromosome orientation on the metaphase plate, relative to the spindle axis, and lagging chromosomes at anaphase that eventually lead to embryonic aneuploidy. However, polar bodies containing DNA are extruded during both meiosis I and II, indicating that while meiotic kinetochores are critical for fidelity some segregation can occur in their absence. We are currently investigating the mechanism of centromeric chromatin-independent meiotic kinetochore assembly as well as the meiotic kinetochore-independent mechanism of chromosome segregation.

1163/B372

The Sgt1-Hsp90 Client-Adaptor Complex Regulates Human Kinetochore Assembly: Screening and Identification of Kinetochore Clients.

A. Davies, K. Kaplan; Molecular and Cellular Biology, University of California Davis, Davis, CA

In budding yeast the Sgt1-Hsp90 client-adaptor complex is required for the formation and stability of the core-kinetochore nucleator CBF3. From yeast to animal cells kinetochore composition is largely conserved with the exception of CBF3. Interestingly, while animal cells lack CBF3, Sgt1 and Hsp90 are conserved and essential for the formation of kinetochores. Inhibition of Hsp90, or depletion of Sgt1, results in the mislocalization of centromeric DNA (CEN DNA) and microtubule proximal kinetochore proteins (Steensgaard et al. 2004, Niikura et al. 2006). However, the specific kinetochore proteins directly regulated by Sgt1 and Hsp90 remain to be identified. In many cases inhibition of Hsp90 results in degradation of its clients (substrates); using this property, we sought to identify human kinetochore clients of the Hsp90-Sgt1 chaperone machine. Neither CEN DNA proximal subunits (i.e., CENP-H, CENP-O, CENP-K or CENP-M) previously shown to mislocalize after inhibition of Hsp90-Sgt1 nor microtubule proximal kinetochore subunits (i.e., Nnf1, Nsl1 and Ndc80) were observed to have altered protein levels following inhibition of Hsp90. In contrast, levels of the central Mis12 component, Dsn1, decreased after inhibition of either Hsp90 or Sgt1. Consistent with Dsn1 being a client of Hsp90, we have identified an Hsp90-Dsn1 complex. The decrease in Dsn1 that results from inhibition of Hsp90 impairs the ability of the Mis12 complex to form and this is sufficient to prevent kinetochore assembly. Our results and previous published observations (Kline et al. 2006) show that loss of Mis12 complex interferes with the assembly of both DNA and microtubule proximal kinetochore complexes and thus mimic the phenotype after Hsp90-Sgt1 inhibition. Together, this data suggest that the Mis12 complex functions as a nucleator of kinetochore assembly, and like the CBF3 complex from yeast, requires the activity of the Hsp90-Sgt1 chaperone machine.

1164/B373

CDK5RAP2 Is Required for Spindle Checkpoint Function.

X. Zhang[1], S. Lv[1], D. Liu[1], X. Zhong[2], B. Liu[1], J. Liao[1], J. Li[1], G. P. Pfeifer[2], X. Xu[1]; [1]College of Life Science, Capital Normal University, Beijing, China, [2]Biology, City of Hope National Medical Center/Beckman Research Institute, Duarte, CA

CDK5RAP2 (cyclin dependent kinase 5 regulatory associated protein 2), when mutated, causes primary microcephaly. It encodes a centrosomal/spindle pole protein. We show here that inhibition of CDK5RAP2 expression causes chromosome mis-segregation, fails to maintain spindle poisons-induced checkpoint, and concomitantly associates with decreased expression of the spindle checkpoint critical protein BUBR1. CDK5RAP2 resides in the BUBR1 promoter and functions as a positive transcriptional regulator. These results suggest that CDK5RAP2 is required for spindle checkpoint function likely through regulation of BUBR1 transcription.

1165/B374

Regulatory Roles of Casein Kinase 2 (CK2) in Mitotic Spindle Function.

Y. Peng, J. Wong, N. Harper, D. Drubin, G. Barnes; Molecular & Cell Biology, Univ of California Berkeley, Berkeley, CA

Mif2 is the budding yeast homolog of human CENP-C. It binds to centromeres and recruits other inner and outer kinetochore proteins. Mif2 is phosphorylated by Ipl1, which is the budding yeast Aurora B kinase. Previously it was determined that a portion of mif2 mutants that cannot be phosphorylated arrest at anaphase. However, the role of Mif2 phosphorylation has not been fully explored. CK2 is a ubiquitous and highly conserved Ser/Thr kinase in eukaryotes. Although CK2 has been linked to a wide variety of cellular processes including cell morphology/polarity, cell survival, apoptosis, proliferation and cell cycle regulation, it has remained largely unclear how CK2 regulates these diverse events. A recent systematic yeast two-hybrid screen focusing on mitotic spindle function identified interactions between Mif2 and two CK2 subunits, Cka2 and Ckb2. In this study, we further investigated the roles of CK2 in mitotic spindle function. Two kinetochore proteins, Mif2 and Cbf2 (Ndc10), were previously identified as putative substrates of CK2. One of two Mif2 phosphorylation sites identified by mass spectrometry is a likely CK2 site. Consistently, we found that yeast Mif2 is phosphorylated by hCK2 in vitro. Moreover, Mif2 phosphorylation is impaired in both ipl1-ts (temperature sensitive) and cka2-ts alleles at non-permissive temperature. We also found that phosphorylation levels of Mif2 oscillate during the cell cycle. It is most highly phosphorylated during anaphase and becomes unphosphorylated as soon as chromosome segregation is complete. These findings suggest that both Ipl1 and CK2 phosphorylate Mif2, and that phosphorylation of Mif2 plays a critical role in chromosome segregation.

1166/B375

Phosphorylation Sites in BubR1 That Regulate Kinetochore Attachment, Tension and Mitotic Exit.

H. Huang[1], J. Hittle[1], F. Zappacosta[2], R. S. Annan[2], A. Hershko[3], T. Yen[1]; [1]Foxchase Cancer Center, Philadelphia, PA, [2]Glaxo Smith Kline, King of Prussia, PA, [3]Unit of Biochemistry, Technion-Israel Institute of Technology, Haifa, Israel

BubR1 kinase is essential for the mitotic checkpoint and also for kinetochores to establish microtubule attachments. We report that BubR1 is phosphorylated in mitosis on four residues that differ from sites recently reported to be phosphorylated by Plk1. S670, the most conserved residue, is phosphorylated at kinetochores at the onset of mitosis and dephosphorylated prior to anaphase onset. Unlike the Plk1 dependent S676 phosphorylation, S670 phosphorylation was sensitive to microtubule attachments but not to kinetochore tension. Functionally, phosphorylation of S670 is essential for error-correction, and for kinetochores with end-on attachments to establish tension. Furthermore, in vitro data suggest that the phosphorylation status of BubR1 is important for checkpoint inhibition of the APC/C. Finally, RNAi experiments show that Mps1 is a major, but not the exclusive kinase, that specifies BubR1 phosphorylation in vivo. The combined data suggests that BubR1 maybe an effector of multiple kinases, that are involved in discrete aspects of kinetochore attachments and checkpoint regulation.

1167/B376

Adaptation of the Spindle Checkpoint.

G. H. Goto[1], A. Mishra[2], R. Abdulle[2], C. A. Slaughter[2], K. Kitagawa[1]; [1]Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN, [2]Hartwell Center for Bioinformatics and Biotechnology, St. Jude Children's Research Hospital, Memphis, TN

During cell division, the spindle checkpoint ensures accurate chromosome segregation by monitoring the kinetochore-microtubule interaction and delaying the onset of anaphase until each pair of sister chromosomes is properly attached to microtubules. The spindle checkpoint is deactivated as chromosomes start moving toward the spindles in anaphase, but the mechanisms by which this deactivation and adaptation to prolonged mitotic arrest occur are not known. We report here that the spindle checkpoint component Bub1 is degraded by the cyclosome or anaphase-promoting complex (APC/C), which helps deactivate the spindle checkpoint in anaphase in the budding yeast. Our results strongly suggest that Cdc28-mediated phosphorylation of Bub1 at T566 is required for adaptation to prolonged mitotic arrest.

1168/B377

SPDL-1 Functions as a Kinetochore Receptor for the Spindle Assembly Checkpoint Protein, MDF-1/Mad1 in Caenorhabditis elegans.

T. Yamamoto[1], S. Watanabe[1], A. Essex[2,3], R. Kitagawa[1]; [1]Molecular Pharmacology, St. Jude Children's Research Hospital, Memphis, TN, [2]Ludwig Institute for Cancer Research, La Jolla, CA, [3]Cellular & Molecular Medicine, University of California, San Diego, La Jolla, CA

The spindle assembly checkpoint (SAC) ensures faithful chromosome segregation by delaying anaphase onset until all sister kinetochores are attached to bipolar spindles. The SAC pathway is mediated by conserved proteins, Mad1, Mad2, Mad3, Bub1 and Bub3, and the molecular mechanism by which these proteins inhibit the anaphase-promoting complex/cyclosome (APC/C) to delay the onset of anaphase is relatively well understood. Mad1 specifically localizes and recruits Mad2 to microtubule-unattached kinetochores to facilitate the efficient binding of Mad2 with Cdc20, an APC/C activator, thereby inhibiting APC/C. However how Mad1 is targeted to the unattached kinetochore remains unclear. A genomewide RNAi screen for synthetic genetic interactors with *san-1/MAD3* and a secondary screen for genes whose depletion caused a defect in activation of the SAC identified *spdl-1*, a C. elegans homolog of Spindly. Unlike the Spindly proteins, which are required to silence the SAC, SPDL-1 is needed to activate it. *spdl-1* genetically interacts with *san-1/MAD3* and the APC/C genes, as *mdf-1/MAD1*

does, suggesting that SPDL-1 functions specifically in an MDF-1/Mad1-mediated pathway. Substantial depletion of SPDL-1 causes chromosome missegregation, leading to embryonic lethality, suggesting that, in addition to the SAC activation, SPDL-1 has a role in chromosome segregation. SPDL-1 localizes to microtubules during mitosis. SPDL-1 also transiently localizes to the kinetochore from prometaphase to metaphase. Kinetochore localization of SPDL-1 depends on KNL-1, a highly conserved kinetochore protein, and CZW-1/Zw10, a component of the RZZ (Rod-Zw10-Zwilch) complex, but not on NDC-80/Hec1, a component of NDC80 complex, or BUB-1. In embryonic cells with monopolar spindles, SPDL-1 localizes to only one side of the kinetochore - the side facing away from the mono centrosome - where MDF-1/Mad1 also localizes in a SPDL-1-dependent manner. In addition, SPDL-1 coimmunoprecipitates with MDF-1/Mad1 in vivo, suggesting that SPDL-1 physically associates with MDF-1/Mad1. These results indicate that SPDL-1 functions as a kinetochore receptor for MDF-1/Mad1.

1169/B378

Bub1 Regulates Chromosome Segregation in a Kinetochore-Independent Manner.

P. Meraldi, C. Klebig; Institute of Biochemistry, ETH Zurich, Zurich, Switzerland

The kinetochore-bound protein kinase Bub1 performs two crucial functions during mitosis: it is essential for spindle checkpoint signaling and for correct kinetochore-microtubule attachment. Interestingly, Bub1 mutations are found in cancer tissues and cancer cell lines. Using an isogenic RNAi complementation system in HeLa cells we investigated the effect of structural Bub1 mutants on chromosome segregation. We demonstrate that Bub1 can regulate chromosome segregation in a kinetochore-independent manner, albeit at lower efficiency. We also identify a novel conserved motif within Bub1, which is located in the central region, that is essential for spindle checkpoint signaling via the recruitment of the checkpoint proteins Mad1 and Mad2, but does not regulate chromosome-microtubule attachment. We show that Bub1 kinase activity is crucial for kinetochore-microtubule attachment and plays a minor role in spindle checkpoint signaling. Finally, we show that several cancer-related Bub1 mutants weaken but do not abrogate the spindle checkpoint, suggesting that checkpoint weakening rather than the loss of checkpoint signaling is implicated in tumorigenesis.

1170/B379

The Kinetochore Protein Mcm21R Regulates Mad2 Stability at Nuclear Pores during Interphase.

S. Chilaka, P. Meraldi; Institute of Biochemistry, ETH Zurich, Zurich, Switzerland

Kinetochores are complex protein machines that link chromosomes to spindle microtubules that are assembled a set of conserved protein-protein interaction networks. One of these protein-interaction network is the 14-subunit CENP-A NAC/CAD complex. We recently showed that CENP-ANAC/CAD subunits fall into two different functional classes: Depletion of Class I proteins (Mcm21RCENP-O and Fta1RCENP-L) disrupts bipolar spindle assembly, while depletion of Class II proteins (CENP-H, Chl4RCENP-N, CENP-I and Sim4RCENP-K) prevents the binding of Class I proteins to kinetochores and impairs chromosome congression, but does not perturb spindle formation. Co-depletion of Class I and Class II proteins restores spindle bipolarity suggesting that Class I proteins regulate or counter-act the function of Class II proteins to drive efficient bipolar spindle assembly (McClelland et al., 2007). Here we tested whether the CENP-ANAC/CAD also regulates the spindle checkpoint. We find that Mcm21RCENP-O or CENP-H depleted cells lack a functional spindle checkpoint and have low Mad2 levels, but normal Mad1, Bub1, BubR1 or Mps1 levels on unattached kinetochores. Interestingly, Mcm21RCENP-O depletion reduces total Mad2 levels by a factor of 10, while CENP-H depletion does not affect Mad2 levels. This indicate that Mcm21RCENP-O stabilizes Mad2 whereas CENP-H is only required for Mad2 loading on kinetochores. Our data also indicate that Mcm21R stabilizes Mad2 not only during mitosis, but also during interphase, and that this stabilization is kinetochore-independent. Based on these results we used a tagged-Mcm21RCENP-O stable cell line to search for potential Mcm21RCENP-O binding partner during interphase. We found that Mcm21RCENP-O interacts with Mad1 and several nuclear pore complex proteins during interphase. Interestingly, Mad2 is known to associate with nuclear pores during interphase. Therefore, we propose that Mcm21RCENP-O is not only a kinetochore protein, but is also associated with the nuclear pores, where it is required for Mad2 stability.

1171/B380

The Nup107-160 Complex and γ-TuRC Regulate Microtubule Polymerization at Kinetochores.

R. Mishra[1], P. Chakraborty[2], A. Arnaoutov[1], B. M. Fontoura[2], M. Dasso[1]; [1]Laboratory of Gene Regulation and Development, NICHD/NIH, Bethesda, MD, [2]Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX

In metazoan cells, the nuclear pore complex (NPC) breaks down during mitosis and many of its constituents are targeted onto spindles, including a sub-complex that will be referred to here as Nup107-160. Nup107-160 is a stable complex that consists of nine NPC proteins: Nup160, Nup133, Nup107, Nup96, Nup85, Nup43, Nup37, Sec13, and Seh1. Nup107-160 is broadly distributed on spindles during prometaphase. It remains kinetochore-bound throughout mitosis, and shows enhanced accumulation on unattached kinetochores. We have found that Nup107-160 interacts with an active form of the γ-tubulin ring complex (γ-TuRC), an essential and conserved microtubule nucleator. Like Nup107-160, γ-TuRC localized to unattached mitotic kinetochores, prompting us to test whether Nup107-160 and γ-TuRC might function in a coordinated manner to promote nucleation of microtubules near mitotic chromosomes and at kinetochores. *Xenopus* egg extracts lacking the Nup107-160 complex or γ-TuRC failed to assemble spindles around sperm chromatin or DNA beads. Moreover, HeLa cells lacking Nup107-160 or γ-TuRC were profoundly deficient in kinetochore-associated microtubule nucleation. Our findings indicate that Nup107-160 promotes spindle assembly through the regulated nucleation of microtubules by γ-TuRC at kinetochores and perhaps other sites within mitotic spindles. These observations suggest an important and novel relationship between the NPC and the microtubule cytoskeleton.

Cell Cycle Controls II (1172 – 1190)

1172/B381

Cloning and Expression of the Human Ultradian Driver of the Cell's Biological Clock.

Z. Jiang[1], S. Dick[1], D. Morre[1,2]; [1]Foods and Nutrition, Purdue University, West Lafayette, IN, [2]Medicinal Chemistry and Molecular Pharmacology, Purdue University, West Lafayette, IN

A homodimeric, growth-related and time-keeping hydroquinone oxidase of the constitutive cell surface with a binuclear copper center and protein disulfide interchange activity with characteristics of an ultradian driver of the biological 24-h circadian clock has been cloned and expressed. The constitutive protein exhibits a recurring complex 2 + 3 set of oscillations in secondary structure, enzymatic activity and redox potential with a period length of 24 min. The period length is temperature independent and entrained by light, melatonin and low frequency EMF. The cDNA has 1929 bp and an open reading frame of 643 amino acids. The gene is located on chromosome 13 (13q 14.11). Functional motifs identified by site-directed mutagenesis include an adenine nucleotide binding site, two copper binding sites and essential cysteines. The recombinant protein expressed in E. coli bound two moles of copper/mole of protein. Bound copper was necessary for activity. The requirement of H260 and H579 both for copper binding and activity was confirmed by site-directed mutagenesis. Addition of melatonin phased the 24-min period such that the next complete period began 24 min after the melatonin addition. Concentrated solutions of the expressed and highly purified protein formed insoluble aggregates devoid of activity resembling amyloid. Mutation of HCKSC120 in the protein changed the oscillatory period length of the protein from 24 min to 30 min. Experiments are in progress with yeast cells transfected with the HCKSC120 mutant to determine the effect of the mutation of the 24-h circadian day.

1173/B382

Staged Assembly of the Histone Gene Expression Machinery at Subnuclear Foci during the Abbreviated Cell Cycle of Human Embryonic Stem Cells.

P. Ghule[1], Z. Dominski[2], X. Yang[2], W. F. Marzluff[2], K. Becker[1], J. W. Harper[3], J. Lian[1], J. L. Stein[1], A. Van Wijnen[1], G. S. Stein[1]; [1]Department of Cell Biology and Cancer Center, University of Massachusetts Medical School, Worcester, MA, [2]Program in Molecular Biology and Biotechnology, University of North Carolina at Chapel Hill, Chapel Hill, NC, [3]Department of Pathology, Harvard Medical School, Boston, MA

Human embryonic stem (hES) cells have an abbreviated G1 phase of the cell cycle. How cells expedite G1 events that are required for the initiation of S phase has not been resolved. One key regulatory pathway that controls G1/S phase entry is the Cyclin E/CDK2 dependent activation of the $p220^{NPAT}$/HiNF-P complex that induces histone gene transcription. In this study, we use the subnuclear organization of factors controlling histone gene expression to define mechanistic differences in the G1 phase of hES and somatic cells using in situ immunofluorescence microscopy and fluorescence in situ hybridization (FISH). We show that histone gene expression is supported by the staged assembly and modification of a unique sub-nuclear structure that coordinates initiation and processing of transcripts originating from histone gene loci. Our results demonstrate that regulatory complexes that mediate transcriptional initiation (e.g., $p220^{NPAT}$) and 3'end processing (e.g., Lsm10, Lsm11 and SLBP) of histone gene transcripts co-localize at histone gene loci in dedicated sub-nuclear foci ('Histone Locus Bodies') that are distinct from Cajal bodies. While appearance of CDK2 phosphorylated $p220^{NPAT}$ in these domains occurs at the time of S phase entry, Histone Locus Bodies are formed ~1 to 2 h before S phase in embryonic cells but 6 h before S phase in somatic cells. These temporal differences in formation of Histone Locus Bodies suggest that the G1 phase of the cell cycle in human embryonic stem cells is abbreviated in part by contraction of late G1.

1174/B383

Anatomical and Morphological Peculiarity of Plant Regeneration from Apical Meristem and Leaf Tissue of Wheat (T>Aestivum L..) In Vitro.

I. Grigina, F. Mukhamedkhanova, T. Matyunina; plant experimental biology, Institute of Genetics, Tashkent, Uzbekistan

At apical meristem study visual valuation showed that morphogenesis on media with 1,5 mg/l 2,4-D was similar ones in immature embryos culture. Shoot forming was on whole callus with large number of regenerants per explant (9/explant). Histological study of these callia preparation showed on the one hand availability of meristematic tissue homogeneous zones, on the other hand, structures that are similar with ones forming from immature embryo scutelum tissue.

1175/B384

Cell Size Control of Asynchronous Nuclear Divisions in Multinucleate Cells.

C. D'Ausilio, A. S. Gladfelter; Biology, Dartmouth College, Hanover, NH

Independent or asynchronous nuclear behavior has been observed in multinucleate cells in checkpoint activation, transcription programs and the nuclear division cycle. We are using the asynchronous nuclear division cycles in *Ashbya gossypii* ("Ashbya") as a model to identify mechanisms for how nuclei behave independently in one cell. *Ashbya* is a filamentous fungus, which is highly related to *Saccharomyces cerevisiae* (95% of genes are homologous and syntenic). However, *Ashbya* undergoes nuclear division without cytokinesis, resulting in one large multinucleate cell and each nucleus within the syncytium divides independently despite access to the shared cytoplasm. The nuclei in an *Ashbya* cell are equally spaced 3-4µm apart. To determine if the cell can control nuclear division timing to maintain this even nuclear spacing, we decreased the nucleocytoplasmic ratio 3-fold with a Nocodazole arrest, which halts nuclear division while still allowing cell growth. When cells are released from this synchronized state with excess cytoplasmic volume, the nuclear division cycle time shortens but remarkably nuclei still divide asynchronously. Thus altered cell size/cytoplasmic volume is translated into different division kinetics while asynchrony persists. This indicates that individual nuclei react unequally to common cytoplasmic proliferation signals. We have observed nuclear volumes range from 3-12μm^3 and thus we hypothesize that variable nuclear division times may arise at least partially from differences in nuclear size. These differences in nuclear volume could be the result of systematic or stochastic asymmetries between related sister nuclei, generated from one mitosis. To distinguish between these possibilities, we are combining time-lapse microscopy and statistical modeling to correlate division times of a large population of sister nuclei. This non-biased, quantitative approach will enable us to determine if asynchrony is a regulated or stochastic feature of the cell cycle in *Ashbya*. In this work we have demonstrated that individual nuclei can respond independently to cell size cues despite residence in one cell and we are testing models for the basis of nuclear autonomous responses to proliferation cues.

1176/B385

Contributions Made by Cdc25 Protein Phosphatases to Embryonic and Adult Cell Cycles in Mice.

G. Lee[1], L. White[1,4], K. Hurov[1,4], T. Stappenbeck[2], H. Piwnica-Worms[1,3,4]; [1]Department of Cell Biology and Physiology, Washington University in St Louis, St Louis, MO, [2]Department of Pathology and Immunology, Washington University in St Louis, St Louis, MO, [3]Department of Internal Medicine, Washington University in St Louis, St Louis, MO, [4]Howard Hughes Medical Institute, St Louis, MO

The CDC25 family of protein phosphatases promotes cell cycle progression by activating cyclin dependent protein kinases. Humans and mice have three family members denoted CDC25A, -B and -C. Two members of this family, CDC25A and CDC25B, are frequently overproduced in human cancers. Disruption of Cdc25B or Cdc25C individually or in combination has no effect on cell cycle regulation or mouse viability. To determine the contributions made by CDC25s to embryonic and adult cell cycles, we generated mice bearing germ-line deletions of Cdc25A and conditional deletions of Cdc25A alone and in combination with Cdc25B and Cdc25C in adult mice. CDC25A null embryos underwent apoptosis during postimplantation development but adult mice conditionally deleted for Cdc25A were indistinguishable from wild-type mice. However, adult mice lacking CDC25B and CDC25C died within one week of Cdc25A disruption due to a lack of small intestinal epithelial cell proliferation. Proliferation loss was accompanied by enhanced Wnt signaling, and concomitant differentiation of progenitors along multiple cell lineages. In particular, crypt base columnar cells, recently identified as intestinal epithelial stem cells, differentiated into Paneth cells. Strikingly, epithelial cells located above the Paneth cell compartment remained undifferentiated and expressed stem cell markers implying that stem cells are retained in this location with loss of cell division. These cells may be the source of recovery after damage to the epithelial lining from sources such as irradiation and chemotherapeutic agents. In summary, CDC25A is the only family member required for early embryonic development in mice. In adult mice, loss of CDC25A can be compensated for by CDC25B and/or CDC25C. Finally, disruption of the entire CDC25 family is lethal in adult mice due to a block in proliferation of small intestinal epithelial cells.

1177/B386

Functional Characterization of Human NDR1&2 as regulators of Rho Family GTPase Signaling Pathways.

K. Magudia, A. Lahoz, A. Hall; Cell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY

NDR (nuclear Dbf2-related) kinases are serine/threonine kinases of the AGC kinase family. There are four NDR kinases in the human genome, which include NDR1, NDR2, LATS1, and LATS2. LATS1&2 are tumor suppressors known to regulate the cell cycle, apoptosis and genomic integrity. Little is known, however, as to how human NDR1&2 function to contribute to cell behavior. Orthologs of human NDR1&2, including Cbk1p (*S. cerevisiae*), Orb6p (*S. pombe*), Sax-1 (*C. elegans*), and Trc (*D. melanogaster*), have been shown to regulate different aspects of cell morphogenetic pathways and cell cycle progression. For instance, the *S. pombe* NDR ortholog, Orb6p, contributes to normal cell shape, regulates polarized growth, and has been implicated in coordinating morphogenesis with cell cycle progression. One study in human cell lines suggests that NDR1&2 may have a role in regulating centrosome duplication. In addition, evidence from microarray data yields examples of both under and over expression of these kinases in different forms of cancer, leaving it unclear as to whether NDR1&2 are oncogenes or tumor suppressors. Rho GTPases regulate the actin cytoskeleton and gene transcription to promote coordinated changes in cell behavior. Interestingly, Sax-1 & Trc mutants have neuronal morphogenesis phenotypes of neurite branching and dendritic tiling that are similar to the effects of dominant negative mutants of Rho GTPases. In addition, Orb6p requires PAK1 activity, a key signaling protein downstream of Cdc42, for its proper subcellular localization. In order to understand the cellular function of NDR1&2 and to define this possible connection to Rho GTPases, I am examining the role of NDR1&2 in cell cycle and morphogenetic pathways in human epithelial cells. Preliminary studies using an RNAi approach show that a decrease in NDR1 expression causes defects in cell proliferation and migration.

1178/B387

Coordinating Growth, Morphogenesis and Cellular Organization of Proliferating Epithelial Tissue.

S. Courty[2], F. Graner[3], J. Friedlander[1], Y. Bellaiche[1]; [1]Developmental Biology, Institut CURIE, Paris, France, [2]LKB, Ecole Normale Supérieure, PARIS, France, [3]Université Grenoble I, Grenoble, France

Although much progress has been made in understanding how the cell cycle regulates tissue size, cell size and number, less is known on how proliferating tissues acquire their shape. Here, we show that the cell texture, which characterizes both cell geometry (cell size and shape) and cell topology (cell arrangement), is regulated during the cell cycle in Drosophila epithelium. Such regulation determines the anisotropic growth of the tissue to control tissue elongation and specifies cell division orientation to control tissue cellular organization. In addition, we show that cell division mis-orientation is compensated by cell-cell rearrangement, establishing that cell division orientation and cell-cell rearrangements are intertwined during the cell cycle to control the organization of the cells within the tissue. Our results identify the cell texture as a novel cell cycle parameter, and define an integrated mechanism by which the cell cycle coordinates the size, shape and the cellular organization of developing epithelia.

1179/B388

Loss of Mitochondrial DNA in the Yeast Crd1 Mutant Leads to Retrograde Pathway Mediated Cell Cycle Arrest.

S. Chen, M. L. Greenberg; Biological Sciences, Wayne State University, Detorit, MI

The anionic phospholipid cardiolipin (CL) and its precursor phosphatidylglycerol (PG) are synthesized and localized in the mitochondrial inner membrane of eukaryotes. They are required for structural integrity and optimal activities of a large number of mitochondrial proteins and complexes. Previous studies showed that loss of anionic phospholipids leads to cell inviability in the absence of mitochondrial DNA (petite lethality). However, the mechanism linking loss of anionic phospholipids to petite lethality was unclear. To elucidate the mechanism, we constructed a crd1Δ rho° mutant, which is viable and mimics phenotypes of pgs1Δ in the petite background. We found that loss of CL in rho° cells leads to cell cycle arrest triggered by induction of Swe1p, a morphogenesis checkpoint protein. Moreover, the retrograde pathway is activated in crd1Δ rho° cells, most likely due to the exacerbation of mitochondrial dysfunction. Interestingly, the expression of SWE1 is dependent on retrograde regulation, as induction of SWE1 is suppressed by deletion of RTG2 or RTG3. Taken together, these findings

indicate that activation of the retrograde pathway leads to cell cycle arrest in crd1Δ rho° cells. These results suggest that anionic phospholipids are required for processes that are essential for normal cell division in rho° cells.

1180/B389

Studying about Neuronal Differentiation of Phosphorylation Site Mutants from Transcription Factor Phox2a.

S. M. Vega Alvarez[1], M. Shin[2], O. Andrisani[2]; [1]Biology, Universidad de Puerto Rico at Mayaguez, San Juan, PR, [2]Purdue University, West Lafayette, IN

Neuronal progenitor cell cycle exit and differentiation has been linked to the transcription and activation of the cell cycle inhibitor p27kip1. p27kip1 inhibits cyclin-dependent kinases that are required for the transition from progenitor cell proliferation to differentiation. The transcription of p27kip1 is regulated by cAMP signaling which activates the homeodomain transcription factor Phox2a. Phox2a is inactive when Ser202, Ser206 and Ser 208 are phosphorylated. cAMP signaling mediates the dephosphorylation of Phox2a at 206, determined by Mass Spectrometry. To investigate the role of Ser202, 206 and 208 phosphorylations in neuronal differentiation, I used a tetracycline-regulated CAD cell line expressing a site directed triple mutant of Phox2a (Ser208/206/202A). The CAD cell line is a cathecholaminergic cell line which differentiates to neurons by cAMP signaling. I hypothesized that the Phox2a triple mutant would not require cAMP-mediated activation, because it cannot be phosphorylated and thus Phox2a will be always active. To address this hypothesis I monitored neuronal differentiation using peripherin immunostaining, a neuronal marker. Employing a time course study, I observe that the tetracycline-regulated cell line expressing the Ser/208/206/202A-Phox2a mutant, in comparison to the wild type (WT) Phox2a-expressing cell line, undergoes neuronal differentiation earlier. In the triple Phox2a mutant long neurites appear within 12h in few cells in the absence of cAMP signaling. By contrast, cAMP signaling induces neurite formation within 5h. Interestingly, in the WT Phox2a-expressing CAD cells neurites appear at a later time, Xh after cAMP stimulation. These results indicate that the triple Phox2a mutant is active without cAMP signaling, although cAMP is probably involved in additional aspects of neuronal differentiation. I conclude that dephosphorylation of Ser 202, 206 and 208 of Phox2a activates this transcription factor required for neuronal differentiation.

1181/B390

Determinants of the Specificity of Plk1 PBD Binding and Development of a Highly Sensitive Plk1 ELISA Assay.

J. Park[1], T. Moulaei[2], D. Lim[3], Y. Kang[1], K. Strebhardt[4], M. Yaffe[3], A. Wlodawer[2], K. S. Lee[1]; [1]NCI, National Institutes of Health, Bethesda, MD, [2]National Cancer Institute-Frederick, National Institutes of Health, Frederick, MD, [3]Departments of Biology and Biological Engineering, Massachusetts Institute of Technology, Cambridge, MA, [4]Dept of Gynecology and Obstetrics, Medical School, J.W. Goethe-University, Frankfurt, Germany

Polo-like kinase 1 (Plk1) plays a pivotal role in the regulation of cellular proliferation. Plk1 is overexpressed in ~80% of human tumors of diverse origins and overexpression of Plk1 promotes neoplastic transformation of human cells. A growing body of evidence suggests that an elevated level of Plk1 expression closely correlates with poor prognosis for a wide range of human cancers. The polo-box domain (PBD) uniquely found in the C-terminal non-catalytic region of Plks plays an essential role in targeting the catalytic activity to specific subcellular locations. The PBD forms a phosphoepitope-binding module and specifically interacts with a phosphorylated motif within its binding target. Here, we report the identification of minimal phosphopeptides derived from the T78 motif of a kinetochores protein PBIP1/Cenp-50 that interacts with the PBD of Plk1, but not the two closely-related Plk2 and Plk3. Comparative binding studies and analyses of the crystal structures of the Plk1 PBD in complex with a minimal phospho-T78 peptide and its derivative revealed that the C-terminal phosphoresidue functions as a high affinity anchor, whereas the N-terminal residues are critical for providing both specificity and affinity to the PBD. Interestingly, Plk1 itself phosphorylates the T78 residue and generates its own PBD-binding site. Using this unique nature of Plk1-dependent phosphorylation and subsequent binding, we developed a rapid, highly sensitive, and specific Plk1 assay that can quantify the levels of Plk1 amount and activity with a small amount of total cellular proteins. With this assay, we demonstrated that Plk1 activity is tightly associated with actively proliferating cells and tissues, and is elevated in tumors but not in normal tissues. The method described here may provide an innovative tool for assessing the predisposition for cancer development, monitoring cancer progression, and estimating the prognosis of patients with cancers from multiple organ sites.

1182/B391

Regulation of Prelamin A Accumulation by the Zmpste24 Endoprotease during Cellular Quiescence.

M. Sinensky, A. Rusinol, C. Bridges; Biochem & Mol. Biol., JH Quillen College of Medicine, Johnson City, TN

Lamin A, is initially synthesized as a 74kDa precursor, prelamin A which undergoes two endoproteolytic cleavages to yield the mature protein. The first cleavage can be carried out by the endoproteases Zmpste24 or Rce1. The second cleavage is unique to lamin A , and is specifically carried out by Zmpste24. The functional significance of this pathway is not understood. We now report that prelamin A accumulates in cells undergoing quiescence. This observation is consistent with reports from other laboratories that cells that do not express A/C lamins do not generally appear competent to enter quiescence under the usual inducing conditions of serum starvation or contact inhibition. This raises the possibility that Zmpste24 regulation of prelamin A accumulation plays a role in quiescence. We now present data consistent with a rapid down-regulation of activity of Zmpste24 in serum-starvation quiescence that correlates with the pattern of down-regulation of DNA synthesis as cells enter quiescence, as well as a rapid increase in activity as serum is returned and DNA synthesis levels increase. In contrast, measurement of the expression levels of Zmpste24 by Western Blot and Northern Blot over a 5 day serum starvation time course shows that the levels of Zmpste24 do not change during the period in which its activity is down-regulated. Therefore, down-regulation of activity of Zmpste24 in quiescence is post-translational. These observations suggest that down-regulation of Zmpste24, presumably to up-regulate prelamin A accumulation, is required to enter or maintain quiescence. In agreement with this hypothesis, we found that expression, by transfection, of Zmpste24 in contact-inhibited fibroblasts prevented cessation of DNA synthesis.

1183/B392

The Effects of an Organosulfuric Polyphenol on Human Melanoma Cell Proliferation and Differentiation.

C. R. Grant[1,2], C. Williams[2], C. Joseph[1], V. Parmar[3], S. T. McKenna[2], A. DePass[2]; [1]Department of Pharmacology, Long Island University, Brooklyn, NY, [2]Biology, Long Island University, Brooklyn, NY, [3]Department of Chemistry, University of New Delhi, New Delhi, India

Coumarins belong to the class of polyphenolic compounds that abundantly occur in the plant kingdom. Many compounds of this class are reported to exhibit different biological activities such as chloretic, analgesic, antispermatogenic and diuretic properties. Consistent with our interest in cell differentiation mechanisms as therapeutic target for cancer, we investigated the anti-cancer properties of VSP-9, a synthetic organosulfur polyphenolic coumarin. We report that when VSP-9 was used to treat the human melanoma cell HO-1, we observed a concentration dependent reduction in proliferation and an induction of differention. Cell viability results and gene expression analyses using indicate that the reduction of proliferation resulted from cell cycle arrest. Morphological changes in the treated HO-1cell population, previously associated with differentiation, in addition to increased melanin expression indicates differentiation of the HO-1 cells in response to sub microgram/ml levels of VSP-9. This reduction on proliferation and induction of differentiation was also accompanied by increases in the expression of the tumor suppressor genes MDA-7/IL-24 (melanoma differentiation gene-7/Interluekin-24) and p53.

1184/B393

Quinoline Derivative KB3-1 Potentiates Paclitaxel Induced Cytotoxicity and Cycle Arrest via Multidrug Resistance Reversal in MES-SA/DX5 Cancer Cells.

Y. Rhee, S. Kim, H. Lee, K. Ahn, K. Ahn, S. Kim; Oriental Medicine, Kyung Hee Univ, Seoul, South Korea

The resistance to chemotherapeutic drugs is a major problem for successful cancer treatment. Multidrug resistance (MDR) phenotype is characterized by overexpression of P-glycoprotein (P-gp) on the cancer cell plasma membrane that extrudes drugs out of the cells. Here we report the discovery of quinoline-3-carboxylic acid [4-(2-{benzyl-3[-(3,4-dimethoxy-phenyl)-propionyl]-amino}-ethyl)-phenyl]-amide (KB3-1) as a novel MDR reversal agent. KB3-1 significantly enhanced the accumulation and retention of a P-gp substrate, rhodamine-123 in the P-gp-expressing MES-SA/DX5 uterine sarcoma cells but not in the P-gp-negative MES-SA cells at nontoxic concentrations of 1 microM and 3 microM. Similarly, fluorescence microscopy observation revealed that KB3-1 reduced the effluxed rhodamine-123 expression on the membrane of MES-SA/DX5 cells. Consistent with decreased P-gp pumping activity, confocal microscopic observation revealed that KB3-1 effectively diminished the expression of P-gp in paclitaxel (TAX)-treated MES-SA/DX-5 cells. Furthermore, Western blotting confirmed that KB3-1 reduced P-gp expression and enhanced cytochrome C release and Bax expression in TAX treated MES-SA/DX-5 cells. In addition, KB3-1 enhanced TAX-induced apoptotic bodies in MES-SA/DX5 cells by TdT-mediated-dUTP nick-end labeling (TUNEL) staining assay as well as potentiated TAX-induced cytotoxicity, G2/M phase arrest and sub G1 apoptosis in MES-SA/DX5 cells but not in MES-SA cells. Interestingly, KB3-1 at 3 microM had comparable MDR reversal activity to 10 microM verapamil, a well-known MDR reversal agent. These findings suggest that KB3-1 can be a MDR reversal drug candidate for combination chemotherapy with TAX.

1185/B394

Characterization of Cell Cycle Mutants in Kluyveromyces lactis.

L. Silveira, L. Fonseca, N. Molinos, B. Yang; Biology Department, University of Redlands, Redlands, CA

Studies of yeast mutants defective in cell cycle progression laid the foundation for much of our understanding of cell cycle regulation. We are extending this successful approach in identifying cell cycle mutants in the yeast *Kluyveromyces lactis*. This screen may identify mutants that were not obtained in other yeasts due to gene redundancy or cellular context. In addition, *K. lactis* mutants enable the study of how lifestyle influences evolution of cell cycle regulation. Like *Saccharomyces cerevisiae*, *K. lactis* is a budding yeast, but its preference for haploidy resembles that of the fission yeast *Schizosaccharomyces pombe*. Division mode and ploidy should exert pressures on cell cycle regulation. For example, *S. cerevisiae* mitotic exit network (MEN) regulators control exit from mitosis, whereas fission yeast MEN homologues regulate septation. Bardin *et al.* (2000; *Cell* 102:21-31) propose that because of their asymmetric mode of division, budding yeast use the MEN to monitor arrival of the nucleus in the daughter cell, whereas fission yeast, which partition down the middle, do not face this problem. Three of ten *ts cdc* mutants have been characterized in detail. Two are MEN mutants, with defects in *CDC14* and *CDC15* homologs. Both display the large buds, segregated DNA, and elongated spindles characteristic of *S. cerevisiae* MEN mutants, rather than the chains of cells seen in cytokinesis mutants. To test whether these mutants were indeed blocked in mitotic exit, Clb2 levels were examined in the arrested *cdc14* mutant. While in wild type cells Clb2 levels fall, in arrested Kl *cdc14* mutants Clb2 levels remain stable, consistent with an inability to degrade Clb2 and exit mitosis. These results buttress the Bardin hypothesis linking division mode to MEN function. A third mutant has an alteration in Kl *TUB2*. Recovery of a beta-tubulin mutant is interesting because Hartwell did not isolate mutants with mitotic spindle defects. Thus, this mutant provides proof of principle that this screen will access a different pool of cell cycle factors than did the previous studies.

1186/B395

Fragile X Mental Retardation Protein and a New Binding Partner Control Cell Cycle Progression during the Midblastula Transition In Drosophila.

O. Papoulas[1], K. Monzo[1], C. Ruse[2], G. T. Cantin[2], J. R. Yates[2], J. C. Sisson[1]; [1]The Section of Molecular Cell and Developmental Biology, The University of Texas at Austin, Austin, TX, [2]The Department of Chemical Physiology, The Scripps Research Institute, La Jolla, CA

Loss of Fragile X mental retardation protein (FMRP) function causes Fragile X Syndrome and autism in humans. FMRP is implicated in the localized translational control of specific mRNAs required for synaptic plasticity in neurons. Despite its profound importance in human cognition we still lack a clear understanding of the mechanism of FMRP function. We have identified two proteins that co-immunoprecipitate with *Drosophila* FMRP (dFMRP) from cleavage stage embryo extracts: eukaryotic initiation factor (eIF) 4G and dFMRP-associated protein (dFAP). eIF4G is essential for translational initiation complex assembly in all eukaryotes and is a major target of translational control. The vertebrate homolog of dFAP is implicated in translational regulation in neurons. In order to study the significance of the dFMRP/dFAP interaction we generated *dfap* antibodies and mutants. Immunofluorescence analysis in fixed wild type embryos reveals extensive co-localization of dFMRP and dFAP to cytoplasmic granules. Their co-sedimentation in sucrose gradients away from polyribosomes suggests they directly regulate translational initiation. Live embryos derived from *dfap-/-*, *dfmr1-/+* double mutant females fail to lengthen the cell cycle normally during the midblastula transition (MBT) and instead undergo a premature mitosis before forming a cellular blastoderm, a

phenotype never observed in *dfap-/-* or *dfmr1-/+* single mutants alone. Western blots reveal normal Cdk1 expression and phosphorylation in double mutants throughout the MBT; however, Cyclin(Cyc)B levels are abnormally high, even during mitosis. Yet, chromosome segregation appears normal in double mutant embryos. Significantly, reducing maternal cycB expression in these mutants suppresses the premature mitotic phenotype. Finally, binding assays show that endogenous dFAP associates with *cycB* mRNA, suggesting it may directly regulate *cycB* mRNA translation. Based on these results we propose that dFMRP functions together with dFAP to repress *cycB* mRNA translational initiation and control normal cell cycle progression during the MBT, and possibly to co-regulate translational initiation of additional mRNAs in neurons later in development.

1187/B396

Role of Human INO80 Chromatin Remodeling Complex in DNA Repair, Replication and Genomic Stability.

S. Hur, E. Park, H. Yoon, J. Lee, J. Kwon; Ewha Womans University, Seoul, South Korea

The INO80 chromatin remodeling complex has recently been characterized to play direct roles in DNA repair and replication as well as transcription in yeast. Although a similar complex exists in mammalian cells, its function has remained largely unexplored. Here, we investigated the cellular functions of the human homolog (hINO80) of yeast INO80 (yINO80). Using small interfering RNA approaches together with a variety of biochemical and cytological techniques, we demonstrate that hINO80 also plays important roles in DNA repair and replication. Data show that hINO80 localizes at replication forks overlapping with PCNA during S phase and that its deficiency leads to defect of DNA replication and S phase progression, suggesting that hINO80 directly participates in DNA replication like yINO80 does. However, the mechanism by which INO80 contributes to DNA repair appears to be different between yeast and human. While yINO80 was shown to directly participate in DNA double strand break (DSB) repair, hINO80 facilitates DSB repair indirectly by stimulating the expression of the genes encoding Rad54 and XRCC3, implicated in DSB repair. Consistent with these results, hINO80 deficiency leads to increase of chromosomal abnormalities under normal conditions as well as after DNA damage. Taken together, these results suggest that hINO80 contributes to the maintenance of genome integrity by stimulating DSB repair via transcription as well as by directly facilitating DNA replication.

1188/B397

Spy1 Regulation of the Intrinsic DNA Damage Response.

C. McAndrew, R. Gastwirt, D. J. Donoghue; Chemistry and Biochemistry, University of California San Diego, San Diego, CA

Human Speedy A1 (Spy1) activates multiple CDKs and its overexpression enhances G1-S phase progression, p27kip degradation, cellular proliferation, and cell survival in response to genotoxic agents, and knockdown of Spy1 renders cells more susceptible to killing by these agents. Spy1 expression also prevents apoptosis and the activation of both S-phase/replication and G2/M checkpoints in UV-challenged cells, as well as the activation of Chk1, RPA, and H2A.X. SAGE data demonstrates that spy1 is one of the fifty most up-regulated genes in nodal metastatic and invasive ductal breast carcinomas. Spy1 overexpression also accelerates mammary tumorigenesis in vivo and leads to abnormal gland morphology during development. We show that Spy1 expression prevents nucleotide excision repair of UV-induced CPDs through bypass of nucleotide excision repair, increases the mutation frequency of UV damaged shuttle DNA, and decreases long-term clonal survival. Spy1 expression also reduces γH2A.X foci formation during the DNA damage response induced by cyclin E overexpression. We also evaluate the effect of Spy1 knockdown on the DNA damage response, and for the first time demonstrate a functional role of endogenous human Spy1. Knockdown of Spy1 by siRNA in cells exposed to UV leads to more γH2A.X foci positive cells compared to control cells. Furthermore, Spy1 knockdown in undamaged cells leads to γH2A.X foci formation, increased Chk1 phosphorylation, and activation of an intrinsic DNA damage response. Knockdown of Spy1 also causes proliferation defects in U2OS cells. The data presented here indicate that Spy1 plays a role in the intrinsic DNA damage pathway by regulating the balance between checkpoint activation, apoptosis, repair, and cell cycle progression in response to stresses and damage inherent to cell growth and proliferation. Spy1 overexpression may tip this balance toward continued cell proliferation, whether a cell experiences stresses from exogenous sources, intrinsic processes, or oncogenic stimulation. Spy1 is an important mediator of the DNA damage response, which is emerging as a crucial cellular mechanism for the regulation of cell growth and oncogenesis.

1189/B398

Taurocholate Feeding to Bile Duct Ligated (BDL) Rats Prevents Caffeic Acid-Induced Bile Duct Damage by Increased Cholangiocyte VEGF Expression.

R. Mancinelli[3,2], P. Onori[5], E. Gaudio[3], A. Franchitto[3], G. Carpino[4], S. DeMorrow[2], Y. Ueno[6], J. Venter[2], G. Alpini[1], H. Francis[7]; [1]Research, Digestive Disease Research Center, Medicine and Systems Biology and Translational Medicine, Central Texas Veterans Health Care System, Scott & White, and Texas A & M Health Science Center College of Medicine, Temple, TX, [2]Medicine, Texas A & M Health Science Center College of Medicine, Temple, TX, [3]Department of Human Anatomy, University of Rome, "La Sapienza", Rome, Italy, [4]Dept Health Science, IUSM, University of Rome, Rome, Italy, [5]Department of Experimental Medicine, State University of l'Aquila, L'Aquila, Italy, [6]Division of Gastroenterology, Tohoku University Hospital, Aobaku, Sendai, Sendai, Japan, [7]R&E and Medicine, Scott & White, and Texas A & M Health Science Center College of Medicine, Temple, TX

Cholangiocytes are the target cells in cholestatic models of ductal hyperplasia including bile duct ligation (BDL). Previously, we have shown that caffeic acid phenethyl ester (CAPE), an active component of propolis from honeybee hives, inhibits cholangiocarcinoma growth by increased apoptosis. In addition, taurocholic acid (TC) protects cholangiocytes against injury induced by parasympathetic or sympathetic denervation by activation of AKT. We also have shown that: (i) cholangiocytes express VEGFR-2 and VEGFR-3; and (ii) VEGF-A and VEGF-C stimulate cholangiocyte growth via an autocrine mechanism. We tested the hypothesis that TC prevents CAPE-induced bile duct damage via changes in cholangiocyte VEGF-A/C expression. Methods: BDL rats (immediately after surgery) were fed 1% TC or control diet in the absence/presence of daily IP injections of CAPE (10 mg/Kg BW). One week later, we evaluated: (i) cholangiocyte apoptosis, and ductal mass in liver sections; (ii) secretin-stimulated ductal secretion; and (iii) VEGF-A/C, VEGF-R2 and VEGF-R3 expression by semiquantitative immunohistochemistry in liver sections. *In vitro*, purified BDL cholangiocytes were stimulated with CAPE (40 μM) in the

presence or absence of TC (40 μM) and with or without pre incubation with a VEGFR-2 or VEGFR-3 inhibitor, before evaluating cholangiocyte apoptosis, proliferation and VEGF-A/C expression. Results: Chronic CAPE administration to BDL rats increased cholangiocyte apoptosis, decreased cholangiocyte proliferation and ductal mass, with the loss of secretin-stimulated ductal secretion. These changes in cholangiocyte growth were associated with reduced expression of VEGF-A/C and VEGF-R2 and VEGF-R3. TC feeding exerted a protective role in CAPE-induced damages, which was associated with enhanced expression of VEGF-A, VEGF-C, VEGF-R2 and VEGF-R3. *In vitro*, TC prevented the CAPE-induced increases in cholangiocyte apoptosis and decreases in biliary proliferation while enhancing the expression of VEGF. These effects were reversed after pretreatment with VEGF receptor inhibitors. Conclusion: Manipulation of cholangiocyte VEGF expression by TC may be important in the regulation of cholangiocyte death/growth in cholestatic liver diseases.

1190/B399

Rev1's Catalytic Activity is Important for 4-NQO Resistance and Induced Mutagenesis in Repair/Tolerance Deletion Backgrounds in S. cerevisiae.

M. Onwugbufor[1, 2], M. Wiltrout[2], G. Walker[2]; [1]Biology, Howard University, Washington, DC, [2]MIT, Cambridge, MA

4-Nitroquinoline-1-oxide is a powerful chemical damaging agent which induces adducts at the N2 position of guanine in S. cerevisiae. These lesions in the DNA template can stall the replication machinery and lead to cell death. Specialized DNA polymerases can help bypass these lesions to enable continuous replication through a process called translesion synthesis. One of these specialized DNA polymerases is Rev1. We know from previous experiments that Rev1's catalytic activity is necessary for resistance to the N2-dG adducts produced by 4-NQO. However, we wanted to better understand the role of catalytic activity for induced mutagenesis. One way to further explore the catalytic dependent mutagenesis induced by 4-NQO was to knock out different repair and damage tolerance pathways. In this project, we knocked out the error-free Mms2 damage tolerance pathway and the error-free Rad14 repair pathway. Both facilitate 4-NQO resistance and influence the frequency of induced mutagenesis. This enabled us to explicitly study the important activity of the catalytic domain in Rev1 for survival and mutagenesis. From the results of our experiments, we concluded that the mutagenesis induced by 4-NQO becomes higher and the survival much more reduced with a catalytically dead Rev1 in the absence of the Mms2 damage tolerance pathway. This further validates the importance of Rev1's catalytic function. However, no significant survival or mutagenesis phenotype was observed.

Mitosis & Meiosis II (1191 – 1220)

1191/B400

CLASP2 Is Phosphorylated During Mitosis.

A. Ramada Maia[1,2], H. Maiato[2,3], I. Kaverina[1]; [1]Cell and Developmental Biology, Vanderbilt University, Nashville, TN, [2]Institute for Molecular and Cell Biology, Porto, Portugal, [3]Laboratory of Cell and Molecular Biology, Faculty of Medicine, University of Porto, Porto, Portugal

CLIP-Associated Proteins (CLASPs) are a family of microtubule plus end-binding proteins widely conserved through species that are critical for mitotic spindle assembly and chromosome segregation. In mammals there are two paralogues mapped to different chromosomes: CLASP1 and CLASP2. These two proteins share high sequence identity and similar localization patterns but are only partially functionally redundant during mitosis. However, regulatory mechanisms that ensure CLASPs function and redundancy are unclear. The aim of our study is to investigate CLASP2 regulation during mitosis and the importance of this paralogue for cell division. Western blot analysis reveals that CLASP2 is specifically phosphorylated during mitosis in cells synchronized by either nocodazole treatment or Eg5 inhibition. No phosphorylation is observed in interphase (G1/S or G2/M) synchronized cultures. Moreover, ectopically expressed CLASP deletion mutants lacking C-terminus do not show phosphorylation indicating that the protein C-terminus is required for mitotic phosphorylation. This is the first observation of cell cycle specific phosphorylation of CLASP, and the first mechanistic evidence of specific regulation of CLASP2 as compared to CLASP1 in mitosis.

1192/B401

Mitotic Arrest, Rather than Spindle Assembly Checkpoint Activation, Is the Direct Trigger for Apoptosis Induced by Anti-mitotic Drugs.

H. Huang, T. Mitchison; Department of Systems Biology, Harvard Medical School, Boston, MA

Anti-mitotic drugs that perturb mitotic spindles are broadly used in cancer therapeutics. Chronic spindle damage induced by anti-mitotics, such as kinesin-5 inhibitors (K5Is), activates the spindle assembly checkpoint (SAC). This arrests cells in mitosis, and for death-sensitive cell lines, leads to apoptotic cell death. Suppression of the SAC attenuates apoptosis induced by anti-mitotics, and thus the SAC has long been speculated to wire directly into apoptotic pathways. However, since SAC inactivation also decreases time spent in mitotic arrest, whether SAC activation triggers apoptosis directly, or indirectly, through mitotic arrest, remains an open question. We established robust mitotic arrest, independent of SAC activity, by depletion of Cdc20 or expression of non-destructible cyclin B. These arrests were insensitive to depletion of SAC proteins (Mad2, BubR1, Mps1 and Bub3). Using single cell analysis by time-lapse microscopy, we showed that in HeLa, a death-sensitive cell line, these arrests caused cell death with kinetics similar to those caused by a K5I drug. Moreover, both types of mitotic arrest triggered mitochondrial apoptosis, as monitored by outer membrane permeabilization and PARP cleavage. Depletion of Mad2, BubR1, Mps1, or Bub3 prevented mitotic arrest and death in the K5I arrest, but in the SAC-independent arrests these depletions had no effect on death kinetics. We conclude that SAC proteins are not required for apoptosis caused by mitotic arrest in HeLa cells. In death-resistant A549 cells, K5I-induced mitotic arrest does not cause cell death; cells slip out of mitosis after 12-16 hrs, and survive as 4N cells. Cdc20 depletion caused mitotic arrest of extended duration in this line, and the cells eventually died after 20-60 hrs of mitotic arrest. Taken together, these results reveal that mitotic arrest, rather than SAC activity, is the direct trigger for apoptosis induced by anti-mitotic drugs, and that exit from mitosis by slippage can protect cells from this death pathway.

1193/B402

Kinesin-5 Inhibition Results in Caspase-dependent DNA Damage during Prolonged Mitotic Arrest, Triggering a Novel and Sustained p53 Response.

J. Orth, A. Loewer, G. Lahav, T. Mitchison; Systems Biology, Harvard Medical School, Boston, MA

Anti-mitotic drugs are used extensively to treat cancers. These drugs cause apoptosis directly from mitotic arrest, or following slippage from mitotic arrest. The tumor suppressor p53 has been implicated downstream of anti-mitotics and is known to be activated by multiple sources of cellular stress, most notably by DNA damage. In order to elucidate the molecular events during and after mitotic arrest, we followed the dynamics of p53 induction in single human cells in response to the specific and potent Kinesin-5 inhibitor (EMD534085) using long-term time-lapse microscopy of fluorescent p53. Rapidly after mitotic slippage, p53 was induced and accumulated in most nuclei. Quantitative analysis revealed two separate classes of p53 dynamics: some cells showed uniform pulses similar to the response to DNA double strand breaks, but most cells showed sustained accumulation of p53 to high levels. Using both comet assays and staining for phosphorylated γH2A.X, we observed high levels of DNA damage late in mitotic arrest and after slippage. Blocking the damage-activated kinase ATM with the inhibitor KU55933 significantly diminished the number and intensity of damage foci in mitotic cells, while the DNA-PK inhibitor NU7026 blocked γH2A.X in post-mitotic cells. What is the source of DNA damage during mitotic arrest? Surprisingly, we found that co-treatment with the caspase inhibitor zVAD-FMK reduced the amount of DNA damage significantly, in both the γH2A.X immunofluorescence and comet assays. Importantly, zVAD-fmk also significantly diminished the induction of p53 in the live-cell imaging experiments. These and additional data indicate that in response EMD534085, cells sufficiently activate caspase(s) during mitotic arrest to initiate DNA damage independently of cell death, resulting in p53 induction that is unique compared to conventional DNA damaging agents. We are currently performing experiments using specific inhibitors, siRNA and biochemical assays to map the precise molecular pathways resulting in the DNA damage and activation of the damage response pathway.

1194/B403

Histone Deacetylase Inhibitors do not Induce Core Histone Hyperacetylation in Metaphase-Arrested HeLa Cells.

J. S. Patzlaff, J. Paulson; Chemistry, University of Wisconsin Oshkosh, Oshkosh, WI

It is known that levels of core histone acetylation are lower during mitosis than during interphase. To explore the reasons for this, we have treated interphase and metaphase-arrested HeLa cells with butyrate and other histone deacetylase (HDAC) inhibitors. As expected, treatment of interphase cells with HDAC inhibitors led to hyperacetylation of the core histones, H2A, H2B, H3 and H4. However, treatment of metaphase-arrested cells in the same way led to very little acetylation of any of the four core histones. Examination of individual acetylation sites using acetylhistone-specific antibodies and immunoblotting revealed that some sites in metaphase chromosomes are acetylated during butyrate treatment and some are not, but no site is acetylated as much in metaphase-arrested cells as in interphase cells. Since histone acetylation is a dynamic process involving both HDACs and histone acetyltransferases (HATs), three possible hypotheses could explain this phenomenon: (1) HDACs are upregulated and/or butyrate-insensitive; (2) HATs are down-regulated; or (3) histone acetylation sites are inaccessible to HATs during mitosis. The first hypothesis can be ruled out, however. In vitro assays indicate that HDAC activity is not increased in mitotic cells. Moreover, treatment of HeLa cells during G2-phase allows metaphase chromosomes to be isolated that contain hyperacetylated histones, ruling out the presence of butyrate-insensitive HDACs. We conclude that the rate of histone acetylation is globally decreased in mitotic chromosomes compared to interphase chromatin, either due to down-regulation of HATs (hypothesis (2)) or chromatin structural changes that make the sites inaccessible (hypothesis (3)).

1195/B404

Sensing Mitotic Duration: Daughter Cells Arrest in G1 after Prolonged but Normal Mitosis.

Y. Uetake, G. Sluder; Cell Biology, UMass Medical School, Worcester, MA

When mitosis is grossly prolonged by microtubule targeting drugs, the cells eventually slip out of mitosis and arrest in G1. The reasons why cells arrest in G1 are not known. To explore this issue, we use normal human cells to characterize the relationship between the duration of mitosis and the ability of the daughter cells to proliferate. Mitosis is prolonged by 3 minutes to 6 hours using low dose nocodazole, Taxol, or monastrol to maintain the activity of the spindle assembly checkpoint. After drug release, the cells satisfy the spindle assembly checkpoint and divide in a normal fashion regardless of mitotic duration. If mitosis is prolonged less than 1 hour, the daughters progress through interphase with normal kinetics and divide again. For prolongations longer than ~1 hour the daughter cells exhibit a durable G1 arrest with elevated p21 expression. The same phenomenon is observed when mitosis is prolonged with the proteasome inhibitor MG132 despite the early satisfaction of the spindle assembly checkpoint. The G1 arrest of daughter cells is not due to DNA damage or lagging chromosomes in anaphase. The G1 arrest is abrogated by the p38 stress kinase inhibitor SB203580 when continuously applied during and after the completion of the prolonged mitosis. These results reveal the existence of a mechanism that senses the duration of prolonged but otherwise normal mitosis and can respond decisively to trigger an irreversible p38 dependent G1 arrest for the daughter cells.

1196/B405

CDC14 Dysfunction Leads to DNA Under-Replication That Is not Detected by Checkpoints.

S. Dulev[1], C. de Renty[2], E. Schwob[2], A. Strunnikov[1]; [1]NIH, NICHD, Bethesda, MD, [2]Institute of Molecular Genetics, CNRS, Montpellier, France

Successful chromosome segregation in mitosis requires the coordinated work of cellular regulatory circuits, in order to transduce the cell cycle signals to chromatin and spindle proteins, which facilitate the restructuring of chromosomes and organize the pulling forces of the spindle. One of such signaling factors is the multifunctional Cdc14 protein, an evolutionary conserved protein phosphatase. The cdc14 mutants arrest in late anaphase with only partially segregated chromosomes. We tested the hypothesis that DNA underreplication prevents completion of chromosome segregation in Cdc14-deficient cells. cdc14 mutants could not stably maintain minichromosomes carrying the rDNA origin of replication, and the rDNA locus was only partially replicated in anaphase. Other genomic regions were also underreplicated in cdc14 mutants, but to a lesser degree than rDNA, as was indicated by DNA combing and ChIP-chip analyses. Slow DNA replication in

cdc14 mutants depends on two synergistic defects: (1) insufficient function of genes directly controlled by MBF or SBF transcription factors containing Swi6p, nuclear import of which requires Cdc14p; (2) a nuclear import defect disrupting stoichiometry of replication factors. An integral view of the cdc14 under-replication phenotype suggests that, due to insufficient amount of replication factors in the nucleus, replication forks are "slow" to replicate the genome and thus cannot complete replication of late replicons. Such a slow replication apparently does not trigger the DNA replication/damage checkpoints, as was previously shown for other mutants.

1197/B406

Rapidity of Cdk1 Activation Determines the Order of Mitotic Events in Somatic Cells.

T. Potapova, G. Gorbsky; CCCB, OMRF, OKC, OK

Mitosis requires a coordinated action of multiple physiological processes in the cell. Cdk1 is the main upstream kinase that directs mitotic progression by phosphorylation of a large number of substrates. Cdk activation displays bi-stability due to positive and negative feedbacks that lock the kinase in a low or high activity state. It is not clear how the switch-like activation of this kinase induces processes that are sequential. Here we show that that the sequence of mitotic events is directed by the dynamics of Cdk activation. We interfered with the molecular components of this feedback-operating system by using chemical inhibitors of Wee1 and Myt1 kinases and Cdc25 phosphatases. Inhibition of Wee1 at the end of the S phase lead to rapid Cdk1 activation and morphologically normal mitotic entry, even though the Cdk activity levels were sub-maximal. Inhibition of both Wee1 and Cdc25 led to not rapid, but continuous activation of Cdk1 limited by availability of a cyclin and slow prophase progression. Unexpectedly, this was followed by morphological and biochemical changes consistent with slipping into pre-mitotic G2. We argue that activating Cdk in this switch-like manner is essential for the ordered mitotic progression in terms of preventing the slippage back to interphase.

1198/B407

Functional interactions of γ-tubulin and the SUMOylation Pathway of Aspergillus nidulans.

E. Szewczyk, C. E. Oakley, B. Oakley; Molecular Genetics, Ohio State University, Columbus, OH

We have identified a SUMO E1 ligase as a multi-copy suppressor of *mipA*D159, a cold-sensitive γ-tubulin mutation of *Aspergillus nidulans* that inhibits growth without inhibiting microtubule nucleation. SUMO is a small ubiquitin-like peptide that influences the activities, interactions and/or subcellular localizations of a diverse array of proteins. To explore the relationship between SUMO and γ-tubulin, we have GFP-tagged and deleted the SUMO gene (*sumO*) and deleted genes that encode other components of the SUMOylation pathway. SUMO localization is dynamic. It is found in punctae in the nucleus in interphase, leaves the nucleus at mitotic onset and re-accumulates in telophase. Deletion of *sumO* (*sumOΔ*) is not lethal but impairs growth slightly. *SumOΔ* causes chromosomal non-disjunction in mitosis and a statistically significant reduction in the length of interphase. *SumOΔ* is synthetically sick with mutant *mipA* alleles, indicating that γ-tubulin and SUMO are involved in the same process (presumably fidelity of chromosomal separation), but operate in different pathways. We have identified and deleted other SUMO pathway genes. Deletions of E1 and E2 ligases phenocopied *sumOΔ*. Deletion of each or both of the E3 ligases or of the homolog of the Ulp1 SUMO protease caused no observable phenotype. Deletion of the Ulp2 homolog caused slight heat sensitivity and deletion of both protease genes resulted in a phenotype identical to *sumOΔ*. Supported by grant GM031837 from the NIH.

1199/B408

The Nuclear Pore Protein Nup153 Has Separable Roles in Both Early Mitotic Progression and the Resolution of Mitosis.

D. Mackay, S. Elgort, K. Ullman; Oncological Sciences, University of Utah, Huntsman Cancer Institute, Salt Lake City, UT

During cell division, accurate inheritance of genomic content requires precise coordination of chromosome dynamics with dramatic rearrangements in cytoskeletal and membrane systems. Proteins that have distinct interphase and mitotic functions provide an important layer in this coordination. We have found that the nuclear pore protein Nup153 functions at two stages during mitosis in mammalian cells. Reduction of endogenous Nup153 levels in HeLa cells results in a delay during the late stages of mitosis accompanied by an increase in unresolved cytokinetic midbodies. Depletion of Nup153 to an even lower threshold leads to an increase in multilobed nuclei and a pronounced defect earlier in mitosis. To address whether the phenotypes observed are different manifestations of the same defect or whether they represent separate functions for Nup153, we used a rescue strategy. This revealed that distinct Nup153 constructs complement the early and late mitotic defects, thus demonstrating that two mitotic functions for Nup153 can be uncoupled. Rescue of the midbody phenotype requires the FG-repeat domain, a region known to contribute to the transport function of the nuclear pore complex. At these levels of Nup153 depletion, however, protein trafficking is not significantly affected, suggesting instead a specialized role for the Nup153 FG-repeat domain that impacts mitotic exit. Together, our results indicate that the nucleoporin Nup153 is critical to the execution of mitosis and plays at least two separable roles at this time of the cell cycle. This study also underscores a growing recognition of nuclear pore complex components as an important resource for mitotic coordination.

1200/B409

A Novel Role in Mitosis for the Complex Containing the Nuclear Factors NF90/NF45.

M. B. Mathews[1], D. Guan[1], R. Shamanna[1], M. Hoque[1], N. Altan-Bonnet[2], D. Lagunoff[3], T. Pe'ery[1,4]; [1]Biochemistry and Molecular Biology, UMDNJ-New Jersey Medical School, Newark, NJ, [2]Biological Sciences, Rutgers University, Newark, NJ, [3]Cell Biology and Molecular Medicine, UMDNJ-New Jersey Medical School, Newark, NJ, [4]Medicine, UMDNJ-New Jersey Medical School, Newark, NJ

Nuclear factor 90 (NF90) and its C-terminally extended isoform, NF110, belong to a family of nucleic acid binding proteins. NF90/110 are isolated with the less-studied protein NF45, as complexes interacting with highly structured and double-stranded RNAs. They have been implicated in the regulation of cellular and viral genes at several levels but it remains unknown whether NF90 and NF110 form functionally distinct complexes. Our objectives were to study the composition of the complexes in vivo and to use siRNA strategies to elucidate their function in cells. NF90 and NF110 both form heterodimeric core complexes with NF45, and are found in cells in large complexes containing multiple NF90/110 isoforms and other proteins. The complexes are dependent on the integrity of NF45, a short-lived protein that regulates the stability of NF90/110. Reciprocally, knockdown of NF90 depletes NF45 as a result of its accelerated degradation. We established HeLa

cell lines that express shRNAs directed against NF45, NF90 or NF110. Cell growth is retarded, and giant multi-nucleated cells accumulate, when NF45 or NF90, but not NF110, is depleted in these cells. The formation of multinucleated cells begins with a defect in completing cytokinesis, followed by cell fusion to form di- or tri-nucleated cells. We observe the formation of anaphase bridges and cells with nuclei that are attached by DNA bridges. Subsequent nuclear divisions of the di-and tri-nucleated cells lead to the formation of multinucleated cells in which nuclei are attached via chromatin. We conclude that NF90/45 complexes regulate cell growth and mitosis. Their depletion results in defects in karyokinesis and cytokinesis. Support: NIH AI034552

1201/B410

The Prometaphase Force-Balance in Drosophila Embryo Mitosis: Requirement for Tightly Regulated Polymer Dynamics and Motor Ratios.

G. E. Civelekoglu Scholey, L. Tao, R. Wollman, I. Brust-Mascher, J. M. Scholey; Molecular and Cellular Biology, University of California, Davis, CA

During prometaphase of mitosis, chromosomes are captured and aligned at the equator. In Drosophila embryos, the prometaphase spindle is maintained at a steady state pole-pole spacing of approximately 8µm before elongating to its metaphase length of 10-12µm. Here we used quantitative modeling of in vitro and in vivo data to test the hypothesis that the steady state length is maintained by a force-balance generated by a kinesin-5 and kinesin-14-dependent antagonistic sliding filament mechanism. Specifically, application of a stochastic force-balance model to data from antagonistic microtubule (MT) gliding assays yielded motor parameters that were then used to model the prometaphase steady state in embryo spindles. We find that a very narrow range of molar ratios of kinesin-5:kinesin-14 is required to maintain a stable prometaphase spindle length. The stability of this steady state length is sensitive to MT dynamic parameters, to changing the oligomeric state of kinesin-5 from bipolar to monopolar, and to the nature of the motors' force-velocity curves in the superstall regime. Finally we find that antagonism between kinesin-5 and kinesin-14 at the steady state balance point is best explained by opposing power strokes rather than by protein friction-induced drag. The results suggest that, in the absence of additional stabilizing factors, the parameters influencing the force generators' dynamic and mechanochemical properties, and the relative concentrations of active motors, must all be fine-tuned in order to maintain a stable prometaphase spindle of constant length.

1202/B411

The Hippo Pathway Effector YAP Has A Role In Mitosis.

D. A. Bui, M. Overholtzer, J. Brugge; Cell Biology, Harvard Medical University, Boston, MA

Yes-associated protein (YAP) is a co-transcription factor that acts downstream of the evolutionarily conserved Hippo pathway. Hippo, Salvador, and Warts, and the mammalian orthologs MST, WW45, and LATS, regulate tissue growth in flies and mammals by sequestering YAP to the cytoplasm to inhibit YAP-dependent transcription. In addition to the conserved functions of this pathway, the mammalian orthologs MST, WW45, LATS also have mitotic roles and may function together as a complex in mitosis, although their precise functions in this context remain unclear. Here we report that YAP is also a mitotic regulator. YAP is modified in mitotic extracts, and localizes to mitotic machinery including the central spindle and midbody ring, where LATS and MST also reside. Knockdown of YAP by shRNA specifically delays abscission, and also induces an anaphase B phenotype associated with increased contractile force that is rescued by inhibition of the Rho-effector Rho-Kinase (ROCK). These data define YAP as a direct mitotic regulator and suggest that the mammalian Hippo pathway proteins, including YAP, function as a mitosis-regulating complex.

1203/B412

Suppression of MAD2 Haploinsufficiency by MAD1 Heterozygosity in Response to Loss of Tension on Mitotic Chromosomes in Budding Yeast.

E. Barnhart, R. Dorer, A. Murray, S. C. Schuyler; Molecular and Cellular Biology, Harvard University, Cambridge, MA

Chromosome segregation depends upon the mitotic spindle checkpoint. The checkpoint promotes accurate chromosome segregation by preventing anaphase progression until microtubule-kinetochore attachments and tension between sister-chromatids have been achieved. Two key components of the checkpoint pathway are Mad1 and Mad2. The Mad1-Mad2 complex is thought to play a critical role by serving as a template to promote the interaction of Mad2 with its target Cdc20, a Cyclosome or Anaphase-Promoting Complex (APC) co-factor required for the metaphase-anaphase transition. It has previously been observed that an increase in Mad1 leads to increased chromosome loss in budding yeast, a defective checkpoint in frog egg extracts, and *MAD2* heterozygous mice display aberrant mitoses and an increase in tumor formation. The goal of this study is to explore the consequences of removing one copy of *MAD2* in the diploid budding yeast *Saccharomyces cerevisiae*. We report here that *MAD2/mad2Δ* heterozygotes display an increase in the rate of chromosome loss. However, *MAD2/mad2Δ* heterozygotes have a normal cell cycle delay in response to treatment with Benomyl, a molecule that depolymerizes microtubules and disrupts microtubule-kinetochore attachments. In contrast, *MAD2/mad2Δ* heterozygotes do not display a checkpoint arrest in response to a lack of tension between sister-chromatids created by the presence of short linear chromosomes. However, both *MAD1/mad1Δ* heterozygotes and *MAD1/mad1Δ MAD2/mad2Δ* double heterozygotes display a normal checkpoint arrest in response to a lack of tension induced by the short linear chromosomes. Finally, we have observed that *MAD2/mad2Δ* heterozygotes do not arrest in response to a loss of tension on all chromosomes induced by a premature loss of sister-chromatid cohesion. We conclude that *MAD2* haploinsufficiency, which leads to an increase in chromosome loss, is due to lack of checkpoint function in response to a loss of tension on mitotic chromosomes. Furthermore, our observation that *MAD1* heterozygosity can suppress *MAD2* haploinsufficiency is consistent with the Mad2-template model, where subunit balance within the Mad1-Mad2 complex is thought to be critical for proper checkpoint function.

1204/B413

Aurora-C Kinase Shares Prometaphase Chromosome Alignment Functions with Aurora-B Kinase.

S. D. Slattery, B. R. Brinkley, R. M. Hall; Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX

Aurora-B kinase regulates major mitotic processes including chromosome condensation, biorientation, and cytokinesis. Aurora-C is the less thoroughly studied homolog of Aurora-B and our understanding of Aurora-C functions in mitotic regulation is incomplete. Aurora-C, like Aurora-B, has been found to be highly overexpressed in some cancer cell lines, and therefore it is important that we fully understand the consequences of aberrant Aurora-C expression in somatic tissues. We have found that Aurora-B and -C share localization patterns, interacting proteins, and substrates. Overexpression of a kinase-defective mutant of either protein lead to cytokinetic failure, while wild-type Aurora-C protein rescued the cytokinetic defects caused by Aurora-B depletion by RNAi. Aurora-C was also able to regulate chromosomal alignment during prometaphase. Aurora-C kinase-defective mutant overexpression caused prometaphase delays characterized by unaligned chromosomes followed by cytokinesis failure, while Aurora-C wild-type overexpression did not cause a delay. In summary, our data shows that Aurora-B and -C share mitotic functions that can be disrupted by aberrant expression patterns that occur in tumor tissues.

1205/B414

Src Kinase and ERM Proteins are Involved in Mitotic Spindle Orientation during Metaphase.

J. Fink[1], A. Betard[2], A. Azioune[1], N. Carpi[1], M. Bornens[1], M. Thery[3], D. Cuvelier[2], M. Piel[1]; [1]UMR 144, Institut Curie, Paris Cedex 05, France, [2]UMR 168, Institut Curie, Paris, France, [3]Biopuces, CEA, Grenoble, France

In the past years we developed an in-vitro system, based on micro-pattern control of extra-cellular matrix proteins geometry, to study how extra-cellular cues control cell division axis (see Thery et al., NCB, 2005 and Thery et al., Nature, 2007). Combining genetics, time-lapse microscopy and biomechanical approaches we have asked wether Src-kinase is involved in proper orientation of cell division axis during metaphase. We could identify three distinct steps in mitotic spindle orientation. Depending on the micro-pattern used, different mechanisms dominate spindle orientation: relative centrosome/nucleus position on isotropic patterns, early alignement along the long cell axis on elongated patterns and late metaphase rotation on patterns with an isotropic shape and an anisotropic cell adhesion pattern. We concentrated on the last step, which was the only one sensitive to Src inhibition: on elongated cells, Src had no effect on spindle orientation, while the metaphase plate did not rotate to orient properly on isotropic patterns with an anisotropic adhesion pattern. To further understand the mechanism by which spindle orientation is controlled during metaphase, we micro-patterned a thin stretchable PDMS film and dynamically changed the micro pattern shape before or during mitosis. We could reproducibly induce spindle rotation during metaphase (while the cell is rounded) by going from an elongated ellipsoid pattern to an orthogonally elongated one by uni-axial stretching. Releasing the stretch also induced spindle rotation back to the initial orientation. These rotations were strongly affected by Src inhibition, showing that extra-cellular cues are dynamically transduced during metaphase, in a Src-dependent manner. We hypothesize that this transduction involves mechano-transduction through retraction fibers linking the cell body to the adhesive micropattern throughout mitosis.

1206/B415

The Role of Kip3, the Yeast Kinesin-8, in Positioning Kinetochores during Metaphase.

M. M. Wargacki, T. Davis; Biochemistry, University of Washington, Seattle, WA

Chromosome congression at metaphase is a conserved feature of mitosis from yeast to humans and depends on the correct assembly and function of a molecular machine called the mitotic spindle. Many molecular motors localize to the mitotic spindle during metaphase and some have been shown to play a role in chromosome positioning. In Saccharomyces cerevisiae, chromosome congression defects can be studied by imaging cells containing fluorophore tagged kinetochore proteins. In wild type cells, sister kinetochores are separated into two clusters on either side of the spindle equator at metaphase. Our data reveal that the yeast kinesin-8 microtubule motor protein, Kip3, is important for properly aligning kinetochores during metaphase. Without Kip3, kinetochores become mis-positioned along the spindle and inter-kinetochore distance is often decreased. These results are consistent with the recently published finding that the human kinesin-8, Kif18A, positions chromosomes by regulating kinetochore microtubule dynamics (Stumpff et al, 2008). We are currently investigating the effect of Kip3 on kinetochore dynamics in yeast.

1207/B416

ATPase Function and Binding of Condensin in Vertebrate Cells.

D. F. Hudson[1,2], S. Ohta[3], W. Earnshaw[3]; [1]Chromosome & Chromatin Research, MCRI, Melbourne, VIC, Australia, [2]Department of Paediatrics, University of Melbourne, Melbourne, VIC, Australia, [3]Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, United Kingdom

Condensin and cohesin are similar ATPase complexes that are essential for mitotic chromosome architecture and segregation. Cohesin is thought to form rings around replicated DNA molecules, but how condensin binds chromosomes is unknown. In both, the role of the ATPase is unknown. This study analyzed condensin ATPase function by systematic mutagenesis of all known SMC2 ATPase domains in SMC2 conditional knockout cells. We prepared a biologically active cleavable form of SMC2, allowing disruption of the putative condensin complex ring in vitro and determination of whether condensin functions like cohesin by topologically embracing DNA. SMC2 ATP binding was required for condensin's stable association with mitotic chromosomes, though not for formation of the condensin complex. Remarkably, disruption of the putative condensin ring didn't affect condensin complex integrity or its association with mitotic chromosomes. Thus, despite their similar molecular organisation, condensin and cohesin apparently exhibit key differences in structure and function.

1208/B417

Haspin Kinase, a Regulator of Cohesion, Phosphorylates Sgo1 as Well as the Chromosome Passenger Complex.

C. D. Jingle, S. Rosasco-Nitcher, T. Stukenberg; Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA

During mitosis cohesion must be maintained between chromatids through metaphase to distinguish sisters, but then quickly lost to segregate chromatids in anaphase. The cohesin complex is deposited during replication and holds sister chromatids together. Although most of the cohesin is removed in prophase by phosphorylation by Polo and Aurora B kinases, cohesion at centromeres is protected by Sgo1. Sgo1 is believed to maintain centromeric cohesion by recruiting PP2a to centromeres where it acts in opposition to Polo and Aurora B kinases. Haspin Kinase is a serine/threonine kinase that has been demonstrated to play a major role in the cohesion of sister chromatids. Haspin

phosphorylates histone H3 on threonine 3, but it is unclear if this event protects cohesion. We have tested and identified new in vitro substrates for haspin kinase. Haspin phosphorylates Sgo1 in vitro. Haspin also phosphorylates the Dasra (Borealin) subunit of the chromosome passenger complex, which contains the Aurora B kinase. Recombinant Haspin on beads can pull down both Sgo1 and Dasra A from Xenopus extracts. We are currently testing whether these events are required to maintain cohesion.

1209/B418

Regulation of Cyclin A Degradation in Mitosis.

B. Di Fiore, J. Pines; Wellcome Trust/Cancer Reaserach UK, Gurdon Institute, Univeristy of Cambridge, Cambridge, United Kingdom

Mitosis is a highly regulated process, during which the genome is equally segregated to two daughter cells. The ubiquitin ligase APC/C plays a crucial role in the regulation of mitotic progression, and by ensuring that specific proteins are degraded at certain time during mitosis it contributes to the order of mitotic events. The APC/C itself is regulated at several levels and in the first part of mitosis its activity is restricted by the spindle assembly checkpoint, a surveillance mechanism which prevents anaphase onset in the presence of improperly attached chromosomes. However, there are early APC/C substrates which are degraded as soon as cells enter mitosis regardless of the presence of an active spindle assembly checkpoint and more generally it is not completely understood how the APC/C is activated at mitotic entry. By using both biochemistry and single cell analysis, we are currently investigating how APC/C activity is regulated at mitotic entry. In particular we are studying the regulation of cyclin A degradation (as one of the early APC/C substrates). We have characterised the region(s) required for its recognition by the APC/C and for its degradation and we are studying whether the difference in the timing of degradation of cyclin A and of later substrates depends on a different recognition by the APC/C or is due to substrate-specific modifications. Furthermore, we also have evidence that cyclin A overexpression affects the degradation of later substrates: we are, therefore, also investigating how cyclin A interferes with the spindle assembly checkpoint.

1210/B419

Separase Is Recruited to Mitotic Chromosomes to Dissolve Sister Chromatid Cohesion in a Polynucleotide-Dependent Manner.

Y. Sun[1], M. Kucej[1], H. Yu[2], H. Zou[1]; [1]Molecular Biology, UT Southwestern Medical Center, Dallas, TX, [2]Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, TX

In mitosis and meiosis, the final separation of sister chromatids is triggered by the separase-catalyzed cleavage of cohesin. Despite that several redundant mechanisms have been identified to restrict the activity of separase in anaphase, our understanding of this process and its regulation remains incomplete. Here we report cohesin cleavage by separase is inhibited by extract prepared from the securin-/- HCT116 cells. Biochemical purification identified the activity as RNase. Further investigations revealed that RNA or DNA is required in the cleavage of cohesin by separase in a sequence-nonspecific manner. Separase binds to DNA, and presumably RNA, in vitro. However, DNA does not stimulate the proteolytic activity of separase, because the auto-cleavage of separase occurs in the absence of any polynucleotide. Instead, biochemical characterizations suggest that separase and cohesin need bind to the same DNA molecule to achieve cohesin cleavage, suggesting that a productive separase and cohesin interaction requires a polynucleotide as the bridge. In HeLa cells, a fraction of separase localizes to the mitotic chromosomes. And in a 293T cell line, stably expressing an inducible V5-tagged separase, separase is detected on the entire length of the mitotic chromosome. Finally, the importance of chromosome in cohesin cleavage is further demonstrated by the observation that the cleavage of the chromosome-associated cohesins is dependent on the presence of chromosomal DNA. This dependence explains why chromosome-associated cohesins are specifically cleaved by separase and the soluble cohesins are left intact in anaphase.

1211/B420

The SCFbTrCP1 is Required for Metaphase Destruction of CDC25B1.

T. Yann, O. Coux, V. Baldin; CRBM-CNRS, Montpellier, France

The cell division cycle 25 (CDC25) family of proteins are highly conserved dual specificity phosphatases that activate cyclin-dependant kinases (CDK), which in turn regulates progression through the cell cycle. When CDK activity is required for progression into the next cell cycle phase, the dual specificity CDC25 phosphatases dephosphorylates the CDK on two residues (Thr14 and Tyr 15), thereby activating the CDK-cyclin complex. One member of this family, CDC25B, which is frequently overexpressed in many cancers, is proposed to be responsible for the initial activation of CDK1-cyclin B at the centrosomes during the G2-M transition. In cells, CDC25B has been shown to be unstable and degraded by the proteasome. To obtain deeper insight into the mechanism of CDC25B degradation, we have investigated the molecular determinants and the exact mechanisms involved in CDC25B destruction in vitro as well as in cells. Here, we show that during the cell cycle CDC25B is completely degraded at the end of mitosis. Using different approaches (cell synchronisation, mutagenesis, protein overexpression,….) we demonstrate that: 1) the degradation of CDC25B occurs during mitosis just after the metaphase/anaphase transition, 2) this degradation depends upon the interaction between CDC25B and the SCFbTrCP1 ubiquitin ligase and 3) the stabilization of CDC25B at this point of the cell cycle induces a delay in mitotic exit, linked to several nuclear defects. Thus, our data highlights the importance of CDC25B degradation for ensuring a proper completion of mitosis.

1212/B421

Mechanisms to Suppress Multipolar Divisions in Cancer Cells with Extra Centrosomes.

M. Kwon[1], S. Godinho[1], N. S. Chandhok[1], N. J. Ganem[1], A. Azioune[3], M. Thery[4], D. Pellman[1,2]; [1]Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, [2]Department of Pediatric Hematology/Oncology, Childrens Hospital, Harvard Medical School, Boston, MA, [3]Biologie du Cycle Cellulaire et de la Motilite, Institut Curie, Paris, France, [4]CEA, Grenoble, France

Effective cancer therapeutics must exploit biological differences between tumor cells and the normal cells from which they arose. One striking feature of many cancer cells is the presence of extra centrosomes. Cells containing multiple centrosomes have the potential to undergo a catastrophic multipolar mitosis that leads to chromosome instability, and potentially cell death. Nevertheless, many cancer cells successfully divide because of mechanisms that suppress multipolar mitoses, most obviously the clustering of supernumerary centrosomes. A genome-wide RNAi screen in Drosophila S2 cells and a secondary analysis in cancer cells defined mechanisms that suppress multipolar

mitoses. In addition to proteins that organize microtubules at the spindle poles, we identified novel roles for the spindle assembly checkpoint, cortical actin cytoskeleton and cell adhesion. Using live cell imaging and fibronectin micropatterns, we found that interphase cell shape and adhesion pattern can determine the success of the subsequent mitosis in cells with extra centrosomes. These findings may identify cancer-selective therapeutic targets: HSET (a Kinesin-14 member), a normally non-essential kinesin motor was essential for the viability of certain extra centrosome-containing cancer cells. These findings define a mechanism by which the tumor microenvironment can potentially influence genome stability and the survival of cancer cells.

1213/B422

Multipolar Spindle Assembly as a Cause of Kinetochore Mis-Attachment and Chromosome Mis-Segregation in Colorectal Cancer Cells.

W. Silkworth[1], I. K. Nardi[1], L. M. Scholl[2], D. Cimini[1]; [1]Biological Sciences, Virginia Tech, Blacksburg, VA, [2]Biology, Oberlin College, Oberlin, OH

Aneuploidy is a ubiquitous feature of cancer cells and many cancer cells display a so called CIN (Chromosome Instability) phenotype, by which they lose or gain chromosomes at each mitotic division. A recent study (Thomspon and Compton, 2008) showed that many CIN cells exhibit anaphase lagging chromosomes (e.g., chromosomes which lag behind at the metaphase plate during anaphase), suggesting this as the underlying mechanism of CIN. Anaphase lagging chromosomes are caused by merotelic kinetochore (KT) orientation (Cimini et al., 2001), a mis-attachment in which a single KT binds microtubules (MTs) from both spindle poles rather than just one. Colorectal cancer cells can be divided in two distinct groups, those with CIN and those with MIN (Microsatellite Instability) phenotype. In this study, we used CIN and MIN colorectal cancer cells to: (i) investigate the mechanism(s) of merotelic KT formation; (ii) unveil other possible mitotic defects contributing to CIN. Using high-resolution fluorescence microscopy on MIN and CIN cells immunostained for KT and mitotic spindle proteins, we found that CIN cells assemble multipolar mitotic spindles early in mitosis at a greater frequency than do MIN cells. These supernumerary spindle poles appear to be functional centrosomes, as shown by the presence of gamma tubulin. Interestingly, these elevated levels of multipolar spindles were not seen in anaphase. Instead, in anaphase higher frequencies of lagging chromosomes were found in bi-polar CIN cells compared to MIN cells. Finally, time-lapse phase-contrast microscopy revealed that there is also an increase in the duration of mitosis in CIN vs. MIN cells. Taken together our data suggest a model by which merotelic KT orientation occurs at higher rates in multipolar CIN cells in early mitosis. Most of these cells would then bi-polarize before anaphase onset, but would not correct all the merotelic mis-attachments, which would result in high frequencies of lagging chromosomes in anaphase. To test this model, we plan to create stable transfected CIN and MIN cell lines with fluorescently labeled MTs and KTs to use in time-lapse fluorescence microscopy experiments.

1214/B423

Tumor Suppression from High Rates of Chromosome Missegregation.

A. Silk[1], S. D. Ryan[2], D. Cleveland[1], B. Weaver[2]; [1]Ludwig Institute for Cancer Research, University of California, San Diego, La Jolla, CA, [2]Pharmacology, University of Wisconsin - Madison, Madison, WI

Aneuploidy, an abnormal chromosome number, is a hallmark of tumor cells. Recognizing this correlation, Boveri proposed aneuploidy to be a cause of tumorigenesis nearly 100 years ago. However, despite its nearly ubiquitous occurrence in malignant cells, a cause-and-effect relationship between aneuploidy and cancer has been difficult to define. This is because most methods of generating aneuploidy produce additional cellular defects that can also promote tumorigenesis, such as DNA damage. We determined that cells and mice with reduced levels of the mitosis-specific, centromere-linked motor CENP-E develop aneuploidy and chromosomal instability without other defects, including DNA damage. CENP-E+/- cells missegregate one or a few chromosomes per division. As Boveri had proposed, the low rate of whole chromosome missegregation caused by CENP-E heterozygosity in the absence of other defects drives an elevated level of spontaneous spleen and lung tumors. However, aneuploidy due to CENP-E heterozygosity suppresses tumors in three different contexts: spontaneous tumors of the liver, tumors caused by treatment with the carcinogen DMBA, and tumors caused by homozygous loss of the p19/ARF tumor suppressor. All three contexts in which CENP-E heterozygosity suppresses tumors have a pre-existing level of aneuploidy that can be increased by depletion of CENP-E. Reduction of CENP-E also causes increased cell death in these contexts. Together, these results support the hypothesis that high rates of chromosome missegregation promote cell death and tumor suppression. We further tested this hypothesis by co-depleting CENP-E and another component of the mitotic checkpoint, Mad2. CENP-E+/-; Mad2+/- double heterozygous cells missegregate more chromosomes per division than CENP-E+/- or Mad2+/- single heterozygous cells and have elevated levels of cell death. CENP-E+/-; Mad2+/- double heterozygous mice exhibit decreased rates of tumor development as compared to mice with reduced levels of CENP-E or Mad2 individually. These findings indicate that while low rates of chromosome missegregation can promote tumorigenesis, higher rates of chromosome missegregation drive cell death and tumor suppression.

1215/B424

Quantitative Analysis of Cdc14 Release Kinetics in Single Live Cells.

S. Ramachandran[1], J. Schwartz[1,2], R. Li[1,3]; [1]Rong Li Lab, Stowers Institute, Kansas, MO, [2]Imaging Center, Stowers Institute, Kansas City, MO, [3]Department of Molecular & Integrative Physiology, The University of Kansas School of Medicine, Kansas City, KS

The division of eukaryotic cell into two daughter cells is regulated by cyclin dependent kinase (Cdk1). In the budding yeast Saccharomyces cerevisiae, Cdc14 play a major role in mitotic exit through two signaling networks, the Cdc14 early anaphase release (FEAR) and the mitotic exit network(MEN), which ensures the proper chromosome segregation between the mother and daughter cell. Cdc14 is required to promote the inactivation of Cdk-Clb pools that remains after anaphase onset. At the onset of anaphase, Cdc14 network become active to promote the release of Cdc14 from the nucleolus. During this time Cdc14 is seen through the nucleus and associates with the spindle pole bodies (SPB) and mitotic spindle. During the late stages of anaphase the MEN become activated and promote further release of cdc14 into the nucleus and also maintain this release. Most of the published studies used immunoflourescence staining to visualize cdc14 in fixed cell. So we done a new approach to quantitative imaging to determine the in vivo dynamics of protein involved in the mitotic exit control. We have built a yeast strain that expresses fluorescently tagged spindle pole body marker (Spc42-macherry), a nucleolus marker (Net1-mcherry) and GFP tagged

Cdc14. The SPB marker allows us to monitor the spindle length, which reflects the progression of anaphase. Time lapse images of live yeast cells are captured using spinning disc confocal microscope and acquired 'z' stacks for every minute. Spindle length and Cdc14 release from Nucleolus is quantified using IMARIS software. Our analysis shows that Cdc14 released from the nucleolus in two distinct steps. It is also interesting to find that Cdc14 coming to the bud neck region six minutes before Myo1 constriction starts. Cdc14 localized always on the spindle pole body which moves to daughter cell and it keeps there even after cytokinesis. It can even witness on SPB, which under going duplication. Localization of Cdc14 on budneck is a good marker for the 2nd step release of Cdc14. Through the different mutants of FEAR and MEN pathway we are predicting the two stage release of Cdc14 from nucleolus.

1216/B425

Synchronizing Chromosome Segregation by Flux-Dependent Force Uniformization at Kinetochores.

I. Matos[1], A. J. Pereira[1], M. Lince-Faria[1], L. Cameron[2], T. Salmon[2], H. Maiato[1,3]; [1]Institute for Molecular and Cell Biology, Porto, Portugal, [2]Biology, University of North Carolina, at Chapel Hill, NC, [3]Cell and Molecular Biology Lab, Faculdade de Medicina, Universitdade do Porto, Porto, Portugal

Every cell division the genetic information compacted into chromosomes must be correctly distributed between two cells. This critical task is mediated by a microtubule-based bipolar structure - the mitotic spindle - which interacts with chromosomes and mediates their synchronous motion towards opposite poles. Bi-oriented chromosomes at metaphase experience centromeric tension as a result of opposing forces acting on kinetochores - the microtubule-attachment sites on chromosomes. Tension has been shown to promote the maturation and correction of microtubule-kinetochore attachments and proposed to signal this information to the spindle-assembly checkpoint (SAC), which controls the degradation of cohesin molecules between sister-chromatids and anaphase onset. However, how the spindle uniformly distributes tension to the different chromosomes in a strongly stochastic environment is not understood. Here we show that centromeric tension uniformity and subsequent anaphase synchrony are promoted by microtubule poleward flux, a conspicuous property of many animal and plant spindles characterized by persistent microtubule translocation towards the poles. The flux-synchrony correlation can be explained by a simple theoretical model in which microtubule poleward translocation events reflect mechanical relaxation of the kinetochore-microtubule interface, allowing locally released tension to be globally redistributed in the spindle. Tension uniformity by flux maximizes the probability that all chromosomes surpass any putative threshold required to satisfy the SAC and promotes the uniform maturation/correction of microtubule-kinetochore attachments, thereby ensuring the subsequent synchronous segregation of chromosomes during anaphase. This is important to prevent the formation of lagging chromosomes, which may lead to aneuploidy and formation of micronuclei or polyploidy due to cytokinesis failure, common cytological features associated with elevated risk of cancer in humans.

1217/B426

Myosin Phosphatase Targeting Subunit1 Controls Mitotic Exit by Antagonizing Polo-like Kinase1.

F. Matsumura, Y. Yamakita, S. Yamashiro; Rutgers, Piscataway, NJ

Protein phosphatases, opposing enzymes of protein kinases, play critical roles in controlling mitosis and cell division. It is, however, not well understood how and which phosphatase counteracts a specific mitotic kinase during mitosis. We have recently reported that myosin phosphatase targeting subunit1 (MYPT1) in complex with its catalytic subunit of protein phosphatase1C antagonizes polo-like kinase1 (PLK1), thereby controlling PLK1-mediated mitotic events (Dev. Cell vol. 14, p787, 2008): Whereas depletion of PLK1 is known to block centrosome maturation and bipolar spindle assembly, leading to mitotic arrest, co-depletion of MYPT1 and PLK1 rescues these phenotypes. Furthermore, depletion of MYPT1 raises phosphorylation of PLK1 at its activating site of Thr210, explaining, at least in part, the antagonism between PLK1 and MYPT1. Here we show that MYPT1 depletion prematurely caused mitotic exit. While control cells are arrested at mitosis by nocodazole or taxol treatment, MYPT1-depleted cells are able to exit from such mitotic arrest. Control HeLa or SW962 cells, when arrested, show strong staining with both anti-cyclin B antibody and anti-phospho(Ser10) histone H3 antibody. In contrast, cyclin B is degraded in MYPT1-depleted cells while phospho (Ser10)-histone H3 staining remains high. Giemsa staining, as well as live cell imaging, revealed that chromatids are precociously separated in MYPT1-depleted cells. Interestingly, MAD2 staining is present at the kinetochores in MYPT1-depleted cells, indicating uncoupling mitotic arrest from the spindle assembly checkpoint. Because phosphorylation of the anaphase promoting complex (APC) by PLK1 has been reported to increase APC activity, these results suggest that un-regulated activation of PLK1 caused by MYPT1 depletion results in premature activation of the anaphase promoting complex, leading to precocious chormatid segregation and mitotic exit.

1218/B427

Anaphase B in Drosophila Embryos: Significance of the Dynamics of Kinesin-5 and Microtubule Plus Ends for Robust Spindle Elongation.

D. Cheerambathur, G. Civelekoglu-Scholey, I. Brust-Mascher, P. Sommi, J. Scholey; Dept. of Molecular and Cell Biology, University of California Davis, Davis, CA

Chromosome segregation involves both chromosome-to-pole motility (anaphase A) and spindle elongation (anaphase B). We show that the steady, linear elongation of Drosophila embryo anaphase B spindles is accomplished by ensembles of highly dynamic kinesin-5 crosslinkers acting on dynamic microtubule (MT) tracks. We generated transgenic strains expressing fluorescent kinesin-5, KLP61F-GFP, in a functional form that supports mitosis and viability. In FRAP experiments, KLP61F-GFP displays dynamic mobility identical to fluorescent tubulin, inconsistent with the binding of kinesin-5 to a static 'spindle matrix' but consistent with a reaction-diffusion model in which most KLP61F is bound to spindle MTs with the remainder diffusing freely. The bound motors appear to take short (≈ 10 steps) runs before detaching again. We find that, at anaphase B onset, KLP61F's MT tracks undergo a cell cycle-regulated spatial reorganization. This involves the establishment of a spatial gradient of MT plus-end catastrophe frequencies, possibly dependent on plus end binding protein, EB1 and length-dependent kinesin-8 activities, which causes interpolar MT (ipMT) plus ends to 'invade' the spindle equator. This produces a robust 'overlap zone' of dynamically unstable antiparallel ipMTs which, without the need for a stable spindle matrix, can be crosslinked and slid apart by

ensembles of transiently binding, modestly processive, kinesin-5 motors, to produce sustained, linear, spindle elongation. (Refs: Cheerambathur et al, 2007, JCB, 177, 995 and 2008, JCB, in press).

1219/B428

A High Throughput Screen for Compounds Causing Forced Mitotic Exit Identifies Two Novel Aurora B Inhibitors.

J. Pouwels[1], A. Salmela[1, 4], A. Varis[2], A. Kukkonen[1], P. Toivonen[1], P. Halonen[1], M. Perälä[1], G. Gorbsky[3], M. Kallio[1, 2]; [1]Medical Biotechnology, VTT Technical Research Centre of Finland, Turku, Finland, [2]Turku Centre for Biotechnology, University of Turku, Turku, Finland, [3]Cell Cycle and Cancer Biology Research Program, Oklahoma Medical Research Foundation, Oklahoma City, OK, [4]Turku Graduate School of Biomedical Sciences, Turku, Finland

The spindle checkpoint ensures the fidelity of cell division by delaying separation of sister chromatids until each chromosome has correct bipolar attachment to spindle microtubules. Interference with spindle checkpoint signaling is considered a promising strategy to treat cancer as it is expected to lead to massive chromosome missegregation followed by cell death. To discover small molecules with anti-mitotic activity, we performed a high-throughput screen (HTS) for compounds that cause premature escape from a mitotic arrest induced by nocodazole, a microtubule destabilizing drug. Altogether, about 70.000 compounds were tested. Here, we report the characterization of two identified hit compounds, the flavonoid fisetin (3,3',4',7-tetrahydroxyflavone) and a compound termed Spindle Checkpoint Inhibitor 2 (SCI2). Both compounds caused premature escape from mitotic arrest induced by nocodazole, taxol (a microtubule stabilizing drug), or monastrol (an Eg5 inhibitor). Importantly, also unperturbed mitotic cells prematurely exited M phase without cytokinesis upon treatment with fisetin or SCI2. The fisetin and SCI2 induced forced mitotic exit was proteasome-dependent as the proteasome inhibitor MG132 prevented escape from nocodazole mitotic arrest. Immunofluorescence analysis showed a significant reduction in the kinetochore accumulation of the key spindle checkpoint proteins BubR1, Bub1, and Cenp-F in fisetin or SCI2 treated mitotic cells compared to controls. Furthermore, fisetin and SCI2 caused delocalization of Aurora B kinase from the inner centromere to chromosome arms in mitotic cells. Interestingly, both fisetin and SCI2 inhibited Aurora B kinase activity in vivo, as shown by reduced kinetochore accumulation of Cenp-A phosphorylated at Ser7, and in vitro (IC50 of 1 µM for fisetin and 5 µM for SCI2). Aurora A and Cdk1 kinase activities were largely unaffected at these drug concentrations. Finally, both compounds induced massive apoptosis within 3 days of treatment. In conclusion, using a novel HTS strategy we have identified two compounds that interfere with the spindle checkpoint through inhibition of Aurora B and that can induce apoptosis.

1220/B429

How to Assemble and Disassemble an Anaphase Inhibitor.

K. G. Hardwick, S. van der Sar, V. Vanoosthuyse, J. Zich; Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, United Kingdom

We use yeast genetics, live-cell imaging, biochemistry and in vitro reconstitution to further our understanding of the molecular basis of spindle checkpoint signalling. A single unattached kinetochore is sufficient to activate the spindle checkpoint, as are attached kinetochores that lack tension. In their presence, the anaphase promoting complex (APC) is inhibited throughout the cell. It remains unclear what signals are emitted by kinetochores, how they are transmitted, and how the APC is actually inhibited. Several putative anaphase inhibitors have been described, such as Mad2-Cdc20, Mad3-Mad2-Bub3-Cdc20 (MCC), and MCC-APC. In fission yeast we can detect all of these complexes in mitosis, but surprisingly we find that their assembly is independent of upstream signalling components, such as Bub1 and Mph1, and can occur independently of kinetochore targeting. We will describe recent experiments on the assembly of these anaphase inhibitors, and assess how their activity is modulated by Aurora and Mps1 kinases. In addition, we will describe roles of certain checkpoint proteins during checkpoint maintenance and during recovery from spindle checkpoint arrest. During recovery from an arrest, cells must ensure efficient bi-orientation of sister kinetochores and then silence the spindle checkpoint. We will describe how cells co-ordinate these processes through the activity of Aurora kinase and opposing phosphatases.

Apoptosis II (1221 – 1238)

1221/B430

p35 Interacts with Organelle Proteins and A-tubulin: Nuclear Translocation of p35 in Dying Cells.

Y. Son, S. Kim, K. Choi, J. Park, H. Kim, B. Rhim, K. Kim; Pharmacology, School of Medicine, Pusan National University, Busan, South Korea

Although it is well documented that p35 from the Autographa californica nuclear polyhedrosis virus prevents cell death by covalently binding to the active sites of caspases in the cytoplasm, we have very limited information concerning the interaction between p35 and non-caspase proteins. We identified heterogeneous nuclear ribonucleoprotein (hnRNP) C1/C2, hnRNP A1, the translocase of the transporter outer membrane 40 (Tom40), and α-tubulin as interaction partners of p35 through mass spectrometry-based functional proteomics using glutathione-S-transferase (GST)-p35 fusion protein as a bait and through pull-down assay using p35-6His, followed by Western blot analysis. The viral protein was localized in the cytoplasm of human cells and at distinct organelles such as the nucleus and mitochondria. In comparison to the nucleus, p35 was more abundant in the cytoplasm, in which it was co-localized with α-tubulin. However, in the cells undergoing apoptotic death induced by actinomycin D, cytoplasmic p35 was translocated into the nucleus, a process that was inhibited by deletions of the N- and C-terminal domains containing leucine-rich motifs. Gene delivery of p35 using recombinant adenoviruses inhibited cytoplasmic compartmentalization of hnRNP C1/C2 and hnRNP A1 in dying cells by actinomycin D. We were able to demonstrate translocation of p35 into the nuclei of dying cells and resultant protection of hnRNPs from redistribution. We propose an active role of p35 in maintaining the integrity of nuclear proteins during apoptosis.

1222/B431

Tools for Eptigenetic Study: Focus on the Change in Science.

Y. Zhou[1], X. Wang[2], L. Ma[1]; [1]R & D, AbboMax Inc, San Jose, CA, [2]Key Lab of Stem Cells Biology, Chinese Academy of Sciences, Shanghai, China

Years or decades, the heredity is written in the language of DNA. Until recently, epigenetic study has blurred the clear picture. The epigenetic processes, include DNA/nuclear protein methylation, acetylation, phosphorylation, ubiquitylation, sumolyation and RNA interference, are involved in a wide variety of diseases, such as almost all the type of cancers, cognitive dysfunction; respiratory, cardiovascular, autoimmune, and psychological illnesses. Thus, an accurate tool for epigenetic study becomes very important. Here, we have generated a panel of tools including antibodies and assays for apoptosis phenomena related to epigenetic changes. Human hepatoma cells hepG2 (1x104/well) were seeded into two of 96-well plates simultaneously. Both plates were treated with 5 µg/ml of camptothecin for 4 hours, The one plate was used for apoptosis assay monitored by the MTT assay, and the other plate was to conduct immuno-fluorescent staining by using a panel of modified histone antibodies, including H3K4[Me1], H3K4[Me2], H3K4[Me3], H3K9[Me3}, ubiquitylation ubH2A, acetylation aceH3K9, phosphorylation phosH3S10. We observed a positive staining on 3T3 by H3K4[Me3], phosH3S10, a weak staining by ubiquitylation ubH2A, H3K9[Me3} and acetylation aceH3K9 which were corresponding to the apoptotic cells, while the higher extents staining (positive) of ubH2A, AceH3K9 on the untreated cells were observed. There was no significant difference in the mono-, di- and tri-methylation of Histone H3K4 among the treated apoptotic cells. The advances of the epigenetic tools we developed will bring a new perspective insight in the field of apoptosis, signal transduction and cancer research, which will help scientist to open another window to test the epigenetic theory in these fields.

1223/B432

Protective Effect of Butin Against Hydrogen Peroxide Induced Apoptosis by Scavenging Reactive Oxygen Species and Activating Antioxidant Enzymes.

R. Zhang, K. Kang, M. Piao, D. Ko, Z. Wang, J. Hyun; Biochemistry, Institute of Medical Science, Cheju National University, Jeju-si, South Korea

The antioxidant property of butin was investigated for cytoprotective effect against H2O2 induced cell damage. This compound showed intracellular reactive oxygen species (ROS) scavenging, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging, inhibition of lipid peroxidation, and DNA damage. This radical scavenging activity of butin protected cell damage exposed to H2O2. Also, butin reduced the apoptotic cells induced by H2O2, as demonstrated by the decreased DNA fragmentation, apoptotic body formation, and caspase 3 activity. In addition, butin restored the activity and protein expression of cellular antioxidant enzymes, superoxide dismutase (SOD) and catalase (CAT) in H2O2 treated cells. Taken together, these findings suggest that butin protected cells against H2O2 induced cell damage via antioxidant property.

1224/B433

Study Photodamage Effect by ClAlPcS2 Sensitizer on B16FO and NIH3T3 Cell Lines by Atomic Force Microscopy and Measurement of Reactive Oxygen Species In Vitro.

K. Tomankova, H. Kolarova; Medical Biophysics, Faculty of Medicine, Palacky University, Olomouc, Czech Republic

High resolution imaging of biological structures and their changes induced by different agents such as drugs we can study by atomic force microscopy (AFM). This technique does not only record surface topography of the biological samples under physiological conditions, but also delivers micromechanical properties at high resolution. For these reasons, we used this relative new microscopic technique to study of the morphology and mechanical properties of the cell lines. We imaged the cells before and after photodynamic therapy (PDT) and sonodynamic therapy (SDT) of photosensitizer ClAlPcS2 by atomic force microscopy. We also compared impact of the photosensitizer onto tumorous and non-tumorous cell lines in combination by measuring the kinetic production of the reactive oxygen species (ROS) during photodynamic therapy. PDT was induced by LED source with total light dose of 15 J.cm-2. To increase the oxidative stress we added SDT by therapeutic ultrasound with intensity 2 W.cm-2, frequency of 1 MHz and time of exposition of 10 minutes insert before light irradiation. As a protection against oxidative stress we used the Silymarin antioxidant and we studied kinetic production of ROS again. The results show kinetic production of ROS within cells during PDT and SDT modification of morphological features investigated by AFM. The tests of viability show fatal effect of the PDT with combination of SDT. On the other hand, Silymarin can lead to an increase of the cell viability after the treatments. Differences in altitude over surface of the cells gave us information about the cell damage and about different component parts of the cell wall. Before PDT individual cells were characterized by smooth surface without protrusion on the whole surface. The combination of sensitizers, specific light source and ultrasound exposition can lead to the loss of surface rigidity and eventually to dramatic changes of the cell shape or to formation of apoptotic body. This work was supported by the Grant Project MSM 6198959216.

1225/B434

Sonodynamic and Photodynamic Therapy on G361 Human Melanoma Cell Line.

H. Kolarova, K. Tomankova, M. Strnad; Medical Biophysics, Faculty of Medicine, Palacky University, Olomouc, Czech Republic

Sonodynamic therapy and photodynamic therapy is a new treatment modality of tumours. The photochemical interactions of senzitizer, light, and molecular oxygen produce singlet oxygen and other forms of active oxygen, such as peroxide, hydroxyl radical and superoxid ion. The tumour is destroyed either by reactive singlet oxygen species (ROS), type II mechanism, and radical products, type I mechanism, generated in an energy transfer reaction. The resulting damage to organelles within malignant cells leads to tumor ablation. Sonodynamic therapy is the use of an agent that is sensitive to ultrasound, allowing deeper penetration and destroying of abnormal cells. Ultrasound-induced cytotoxicity of sonochemical sensitizers inhibits tumor growth In this study disulfonated chloroaluminum phthalocyanine was selected for testing as a potential sensitizer for combination of sonodynamic and photodynamic therapy.We report the production of reactive oxygen species on G361 melanoma cells. The production of ROS was investigated by molecular probe CM-H2DCFDA. The light emitting diodes (LEDs 670 nm, FWHM 15 nm, 10 mW.cm-2)) were used as a source for evocation of the photodynamic effect. Ultrasound generator with transducer area 4cm-2, frequency 1 MHz and intensity 2 W.cm-2 was used for evocation of sonodynamic effect. Cell damage was evaluated using fluorescence microscope microscopy. The quantitative ROS production changes in relation to sensitizer concentration, irradiation doses and

ultrasound intensity were proved by fluororeader. Ultrasound treatment can support the photodynamic effect because sensitizer can be relocalizated in the cells. Efficiency of photodynamic therapy and sonodynamic therapy is affected by a number of factors including absorption spectrum of the photosensitizer, wavelength of the activation light, depth of the light and ultrasound penetration in the biological tissue, tissue answer on singlet oxygen. Our results indicate a synergistic effect of chloroaluminium phthalocyanine, light and ultrasound on reactive oxygen species production in G361 melanoma cells. This work was supported by the Ministry of Education of the Czech Republic MSM 6198959216.

1226/B435

Apoptosis Induced by Endoplasmic Reticulum Stress Involved in Chronic Ocular Hypertension Model.

J. Kim, Y. Lee, O. Hong, C. Park; Ophthalmology, College of Medicine, The Catholic University of Korea, Seoul, South Korea

This study investigated the involvement or ER-associated apoptosis in glaucoma of chronic ocular hypertension rat retinas. Chronic ocular hypertension was induced in one eye of rats by cauterizing three episcleral vessels. We performed TUNEL, immunohistochemistry, western blot, and real time RT-PCR to analyze indicators of ER molecule chaperone and ER-associated apoptosis. After ocular hypertensive occlusion, TUNEL-positive cells were detected in the retinal ganglion cell layer (GCL) and these gradually increased in the retina. The ER stress sensors including glucose regulated protein-78 (GRP78/BiP), phospho-pancreatic ER kinase (PERK), phospho-eukaryotic initiation factor 2 (p-eIFα2), C/EBP-homologous protein (CHOP) were significantly up-regulated in a time-dependent manner. Furthermore, p-PERK immunoreactivity was observed in GCL and inner nuclear layer (INL). CHOP immunoreactivity was also observed in GCL. Taken together, those results indicated that the ER stress-mediated apoptosis pathway may contribute to ganglion cell death in the retina after chronic ocular hypertension.

1227/B436

Nuclear Localization and Pro-Apoptotic Signaling of YAP2 Require Intact PDZ-Binding Motif.

T. Oka, M. Sudol; Weis Center for Research, Danville, PA

YAP2 (Yes-associated protein, isoform 2) is a WW domain-containing transcriptional co-activator that regulates expression of a specific repertoire of genes by direct binding to several transcription factors. Both YAP2 and YAP1 are major effectors of the mammalian Hippo signaling pathway that controls organ size. YAPs have ability to translocate from cytoplasm to nucleus. Recently, we reported that YAP2 has an ability to bind and stabilize pro-apoptotic member of the p53 family, p73, and that the YAP2-p73 complex promotes apoptosis. We also showed that the cytoplasmic localization of YAP2 is controlled by 14-3-3 protein and that Lats1 kinase phosphorylates YAP2 at Serine-127. The phospho-Serine-127 is a part of peptide motif that binds to the 14-3-3 anchor and sequesters YAP2 in the cytoplasm.YAP2 has also a PDZ-binding motif at its COOH terminus. As PDZ domains are frequently present in sub-membraneous scaffold proteins and we wanted to examine if YAP2 localization in cells is PDZ dependent. To analyze the role of the PDZ binding motif in YAP2, we generated delta C mutant of YAP2 lacking five most COOH terminal amino acids, which constitute a well-conserved PDZ-binding motif. Interestingly, we found that the PDZ domain-binding motif was: (i) necessary for YAP2 localization in the nucleus as the delta C mutant of YAP was found only in the cytoplasm and was excluded from the nucleus; (ii) indispensable for the stabilization of p73; and (iii) crucial for promoting apoptosis. Our data also documented a correlation between the nuclear localization of YAP2 and the stabilization of p73, showing that both are required for the apoptotic function of YAP2. We suggest that an unknown PDZ domain-containing protein(s) functions as a shuttle facilitating YAP2 translocation from the cytoplasm to the nucleus.

1228/B437

7, 8-Dihydroxyflavone Protects Hydrogen Peroxide Induced DNA Damage in Lung Fibroblast Cells.

M. Piao, R. Zhang, K. Kang, D. Ko, Z. Wang, J. Hyun; Department of Biochemistry, School of Medicine, Institute of Medical Science, Cheju National University, Jeju-si, South Korea

We elucidated the protective effect of 7, 8-dihydroxyflavone against hydrogen peroxide (H2O2)-induced DNA damage. Through fluorescence spectrometery, we found that 7, 8-dihydroxyflavone scavenges the intracellular reactive oxygen species (ROS). In effect, 7, 8-dihydroxyflavone prevented the H2O2-induced cellular DNA damage, as evidenced by comet tail, 8-hydroxy-2 deoxyguanosine (8-OHdG) content, and phospho-histone H2A.X expression. Hence, 7, 8-dihydroxyflavone was shown to protect cell via the inhibition of apoptosis induced by H2O2. This was substantiated by decreased apoptotic nuclear fragmentation, decreased sub-G1 cell population, and decreased DNA fragmentation. Furthermore, 7, 8-dihydroxyflavone activated the Akt signal pathway, which is a major survival signal pathway. In addition, the Akt specific inhibitor (LY294002) attenuated the protective effect of 7, 8-dihydroxyflavone against H2O2-induced cell damage. In conclusion, 7, 8-dihydroxyflavone was shown to possess cytoprotective properties against oxidative stress by scavenging intracellular ROS and enhancing Akt activity.

1229/B438

Protective Effect of Esculetin Against Oxidative Stress Induced Cell Damage via Scavenging Reactive Oxygen Species.

K. Kang, R. Zhang, M. Piao, D. Ko, Z. Wang, J. Hyun; Department of Biochemistry, School of Medicine, Institute of Medical Science, Cheju National University, Jeju-si, South Korea

Aim: To investigate the antioxidant properties of esculetin (6, 7-dihydroxycoumarin) against H2O2-induced Chinese hamster lung fibroblast (V79-4) damage. Methods: The radical scavenging activity was assessed by 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radical, hydroxyl radical, and intracellular reactive oxygen species (ROS). In addition, lipid peroxidation was assayed by the measure of related substances which react with thiobarbituric acid. The amount of carbonyl formation in protein was determined using a protein carbonyl ELISA kit. As well, cellular DNA damage was detected by western blot and immuno-fluorescence image. Cell viability was assessed by 3-(4, 5-dimethylthiazole-2-yl)-2, 5-diphenyltetrazolium bromide assay. Results: Esculetin exhibited DPPH radical scavenging, hydroxyl radical scavenging, and intracellular ROS scavenging activities. The radical scavenging activity of esculetin resulted in the protection of cells from lipid peroxidation, protein carbonyl, and DNA damage induced by H2O2. Therefore, esculetin recovered cell viability exposed to H2O2. Conclusion: Esculetin

efficiently attenuated the oxidative stress induced cell damage via its antioxidant properties. As a result, esculetin may be useful in the development of functional food and raw materials of medicine.

1230/B439

Inhibtion of Cyclooxygenase-2/Prostaglandin E2 Pathway Potentiates Icariside II Induced Apoptosis in Human PC-3 Prostate Cancer Cells.

S. Kim, K. Ahn, K. Lee, H. Lee, K. Ahn, S. Kim; Oriental Medicine, Kyung Hee Univ, Seoul, South Korea

Icariside II (3, 5, 7-trihydroxy-4'-methoxyflavone-3-O-a-L-rhamnopyranoside) isolated from the roots of Epimedium Koreanum Nakai was known to have antioxidant activity and inhibit melanogenesis and hypoxia inducible factor alpha. Here, we for the first time report the apoptotic mechanism of icariside II in human PC-3 prostate cancer cells. Icariside II exerted cytotoxicity against PC-3 cells with IC50 of approximately 20 μM. Icariside II suppressed both constitutive and arachidonic acid (AA)-induced cyclooxygenase-2 (COX-2) expression as well as reduced prostaglandin E2 (PGE2) levels in PC-3 cells even at a low concentration of 10 μM. However, icariside II increased sub G1 apoptotic portion and exhibited terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL)-positive apoptotic bodies in PC-3 cells at 20 μM. Furthermore, icariside II attenuated the mitochondrial membrane potential, released cytochrome C into cytosol, activated caspase-9, -8, and -3 expression and cleaved poly (ADP-ribose) polymerase (PARP) in PC-3 cells. Taken together, these findings demonstrate that icariside II inhibits COX-2 and then induces apoptosis mainly via mitochondrial dependent pathway as a potent cancer chemopreventive agent.

1231/B440

A Nucleocytoplasmic Malate Dehydrogenase Regulates p53 Transcriptional Activity in Response to Metabolic Stress.

S. Lee[1,2], J. Kim[1,2], H. Youn[1,2]; [1]Biochemistry and Molecular Biology, Seoul National University College of Medicine, Seoul, South Korea, [2]Biomedical Science, Seoul National University Collegy of Medicine, Seoul, South Korea

Metabolic enzymes have been shown to function as transcriptional regulators. p53, a tumor-suppressive transcription factor, was recently found to regulate energy metabolism. These combined facts raise the possibility that metabolic enzymes may directly regulate p53 function. Here, we discover that nucleocytoplasmic malate dehydrogenase-1 (MDH1) physically associates with p53. Upon glucose deprivation, MDH1 stabilizes and transactivates p53 by binding to p53-responsive elements in the promoter of downstream genes. Knockdown of MDH1 significantly reduces binding of acetylated-p53 and transcription-active histone codes to the promoter upon glucose depletion. MDH1 regulates p53-dependent cell cycle arrest and apoptosis in response to glucose deprivation, suggesting that MDH1 functions as a transcriptional regulator for a p53-dependent metabolic checkpoint. Our findings provide insight into how metabolism is directly linked to gene expression for controlling cellular events in response to metabolic stress.

1232/B441

The Metabolic Sensor C-terminal Binding Protein (CtBP) Maintains Mitochondrial Activities.

J. Kim[1,2], H. Youn[1,2]; [1]Biochemistry and Molecular Biology, Seoul National University College of Medicine, Seoul, South Korea, [2]Biomedical Science, Seoul National University, Seoul, South Korea

Glucose is a primary carbohydrate source of living cells and is converted to other metabolites which are necessary to cellular activities. Therefore, glucose homeostasis is the most important in cells and tightly regulated in the body. However, metabolic stress such as the unbalance of glucose homeostasis triggers a variety of cellular responses, cellular growth and gene expression. As a metabolic center, mitochondria play an essential role that is responsible for cellular energy production and cell death in response to various stimuli. Therefore, the change of cellular energy state affects mitochondrial activities. C-terminal binding protein (CtBP) has been known to be a transcriptional corepressor that interacts with a variety of transcription factors which are involved in the development and tumorigenesis. In addition, CtBP is a member of large transcriptional repressor complex. Structurally, CtBP shares substantial amino acid homology with NAD+(H)-dependent 2-hydroxyacid dehydrogenase and has intrinsic dehydrogenase activity. Theses previous reports raised the possibility that CtBP serves as a metabolic sensor for linking between the cellular energy state and gene transcription. In this study, we found that CtBP represses Bax transcription by binding to E-box region in its promoter in normal glucose condition. Glucose withdrawal induces the dissociation of CtBP from Bax promoter and significant changes of histone codes in Bax promoter. In addition, ctbp knockout leads to an increased Bax expression in normal glucose condition. As a result, we observed the translocation of Bax protein to mitochondria and the change of mitochondrial morphology, such as mitochondrial fusion and hollow cristae structure in ctbp knockout cells. Moreover, we found that reduced cellular ATP level and low oxygen consumption rate in ctbp knockout cells. However, stable expression of CtBP1 and knockdown of Bax mRNA in ctbp knockout cells recovered mitochondrial morphology, suggesting that CtBP functions as a metabolic sensor that maintains mitochondrial activities. Our findings provide new insight into both transcriptional regulation by metabolic stress and its effects on cellular activities.

1233/B442

Role of Caspase-8 on Resveratrol-Induced Apoptotic Cell Death in Rheumatoid Synovial Fibroblasts.

B. Choi, H. Byun, M. Won, K. Park, Y. Kim, Y. Li, H. Lee, J. Hong, J. Seok, G. Hur; Pharmacology, Chungnam National Univ., Daejeon, South Korea

Resveratrol exhibits strong antiproliferative and anti-inflammatory properties. Although recent studies have provided support for the use of resveratrol in human cancer chemoprevention, the effect of resveratrol on the cellular death signaling in rheumatoid arthritis (RA) has never been assessed. This study was conducted to evaluate the antiproliferative potential of resveratrol against primary human synovial fibroblasts of RA (SFRA). Treatment with resveratrol resulted in a dose- and time-dependent apoptotic cell death on SFRA and loss of mitochondrial membrane permeability (MMP) as measured by a rhodamine123. p53 null mouse embryonic fibroblasts (MEFs) also showed the similar rate of apoptosis and loss of MMP, compared to that of wild type MEFs, suggesting that resveratrol induces an apoptotic cell death in a p53-independent manner. Furthermore, our data showed the resveratrol-treatment resulted in significant activation of caspase-8. Our data

suggested that resveratrol causes an activation of caspase-8, in turn, results in modulations in mitochondrial death pathway to lead an active apoptosome complex. This work was supported by Korea Science & Engineering Foundation through the Infection Signaling Network Research Center (R13-2007-020-01002-0) at Chungnam National University and grant from the National R&D Program for Cancer Control Ministry of Health & Welfare, Republic of Korea (No: 0720560).

1234/B443

Oxidative Stress-Activated Calpain and Caspase 3-Mediate DNA Damage and Apoptosis via ATM Degradation in Pancreatic Acinar AR42J Cells.

S. Cho[1], J. Lim[2], K. Kim[1], H. Kim[3]; [1]Department of Pharmacology, Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, South Korea, [2]Institute of Gastroenterology, Yonsei University College of Medicine, Seoul, South Korea, [3]Department of Food and Nutrition, Brain Korea 21 Project, Yonsei University College of Human Ecology, Seoul, South Korea

Degradation of ATM and apoptotic cell death linked to oxidative DNA damage have been implicated in acute pancreatitis. The severe DNA damage, which is beyond the capacity of the DNA repair proteins, triggers apoptosis. The most deleterious form of DNA damage is double-strand DNA breaks (DSBs), where ataxia telangiectasia mutated (ATM) is the main transducer of the double-strand DNA break signal. It was hypothesized that oxidative stress may induce the DNA damage and apoptosis of pancreatic acinar AR42J cells through degradation of ATM. However, the proteases which are involved in degradation of ATM have not been clarified. In the present study, we investigated whether the glucose oxidase acting on β-D-glucose (G/GO), which continuously produces H_2O_2, induce DNA damage and apoptosis via calpain-caspase-madiated ATM degradation. We here found that degradation of ATM, and activities of calpain and caspase-3 were increased by G/GO. Calpain inhibitor calpeptin and caspase-3-specific inhibitor z-DEVD suppressed DNA damage and apoptosis, as well as ATM degradation in G/GO-treated cells. These inhibitors also suppressed G/GO-induced increase of Bax and decrease of Bcl-2 expressions. In conclusion, oxidative stress induced DNA damage and apoptosis through ATM degradation, which were increased by activation of calpain and caspase-3. Proteolysis of ATM by protease including calpain and caspase-3 may underlie the mechanism of apoptosis in pancreatic acinar cells after oxidative stress.

1235/B444

Positive Regulatory Role of SGK-1 on the ERK Function during Liver Regeneration.

M. Won, K. Park, H. Byun, Y. Kim, B. Choi, H. Lee, Y. Li, J. Hong, J. Park, J. Seok, G. Hur; Department of Pharmacology, Chungnam National University, College of Medicine, Daejeon, South Korea

it has established that mitogen-activated protein kinase kinase (MEK)/extracellular signal-regulated kinase (ERK) signaling pathway plays a crucial role in the process of liver regeneration, but the molecular mechanism of ERK activation have remained unclear. We report here that serum- and glucocorticoid- inducible kinase 1 (SGK1) is dynamically up-regulated during liver regulated during liver regeneration and activates ERK signaling by promoting the MEK/ERK complex formation. Partial hepatectomy (PH) resulted in the biphasic transcriptional induction of SGK1 with a concomitant dual phosphorylation of ERK in regenerating liver tissues. Infection of primary hepatocytes and in vivo rat liver with an adenoviral vector encoding constitutively active SGK1 (SGK1, T256D) enhanced the ERK phosphorylation under serum-starved condition and this was blocked by the expression of kinase-dead SGK1 (SGK1, K127M). SGK1 was found to physically interact with ERK1/2 as well as MEK1/2 in co-immunoprecipitation assay and its association was enhanced in the presence of the serum. Importantly, SGK1 mediated the phosphorylation of ERK2 on Ser29 in a serum dependent manner, and mutation of this amino acid with alanine blocked phosphorylation by activated SGK1. Furthermore, replacement of Ser29 to aspartic acid, which mimics the phosphorylation of Ser29, enhanced the basal and serum-induced ERK2 activity as well as the MEK/ERK complexes formation. Taked together, these results suggest that the SGK1 expression during the liver regeneration is a part a signaling pathway that is necessary for enhancing ERK signaling activation through the modulating the MEK/ERK complex formation. This work was supported by a grant from the National R&D program for Cancer Control Ministry of Health & Welfare, Republic of Korea (No: 0720560), and FG 06-2-17 of the 21C Frontier Functional Human Genome Project from the Ministry of Science & Technology in Korea.

1236/B445

Effect of Sulforaphane on Proteomic Pattern and Apoptosis in LNCaP Cells.

S. Won, C. Lee, S. Kim, H. Lee, H. Lee, K. Ahn, S. Kim; Oriental Medicine, Kyung Hee Univ, Seoul, South Korea

Sulforaphane, an isothiocyanate naturally found in cruciferous vegetables, was known to inhibit the proliferation of LNCaP and PC-3 prostate cancer cells and induces G2/M arrest in DU145 cells. Nevertheless, the effect of sulforaphane on protein patterns in LNCaP cells still remains unclear. Thus, to identify the molecular targets of sulforaphane in LNCaP cells, two-dimensional gel electrophoresis (2-DE)-based comparative proteomics was performed and altered proteins in LNCaP cells exposed to sulforaphane were identified by Ettan MALDI-TOF. Proteomic analysis revealed that sulforaphane differentially regulates nine protein spots with over 4-fold increased or decreased expression compared with untreated control. Increased proteins were identified as melanoma-derived leucine zipper containg extra-nuclear factor (MLZE), activin A type I receptor precursor(TGF-β family) and one unnamed protein, whereas decreased proteins were tubulin beta 2, phosphoglucomutase 3 (PGM3), Smoothelin-A, KIAA0073, hypothetical protein LOC57691 and one unnamed protein. Among them, Smoothelin-A, KIAA0073, hypothetical protein LOC57691 were novel proteins. Furthermore, sulforaphane effectively activated caspase 3 and cleaved poly (ADP-ribose) polymerase (PARP) in LNCaP cells by Western blotting. Taken together, our findings suggest that sulforaphane can alter protein expression and induce apoptosis in LNCaP cells

1237/B446

Loss of Pnn Induces ASF/SF2-mediated Apoptosis in MCF-7 Cells through Expression of Bcl-Xs.

S. Leu[1], P. Ouyang[1,2]; [1]Dep. of Anatomy, Chang Gung University, Guei-San, Tao-Yuan, Taiwan, [2]Molecular Medicine Research Center, Chang Gung University, Guei-San, Tao-Yuan, Taiwan

Objective: Pinin (Pnn), a SR related protein, has been shown to play multi-functional roles within eukaryotic cells including cell-cell adhesion, cell migration, regulation of gene transcription, mRNA export, and alternative splicing. Our previous study demonstrated that loss of pnn induces mouse embryonic lethality, however, the underlying mechanism was still unknown. Therefore, we generated a RNAi based cultured cell model to reveal the biological role of pnn in apoptosis. Methods: MCF-7 and HUVEC cells were transfected with siRNA oligo duplex or shRNA vector combining EGFP expression to knockdown Pnn expression. For overexpressing ASF in pnn-depleted cells, DsRed-ASF was constructed with AAV system. Total RNA and whole cell lysates were extracted 48 hours post-transfection and carried out with RT-PCR and Western blot. Results: Cellular apoptosis and nuclear condensation triggered by pnn-depletion were observed in cultured MCF-7 cells and confirmed with immunostaining. With pnn-depletion, nuclear speckles were disrupted and expression levels of SR proteins were diminished. RT-PCR analysis on apoptosis and mRNA splicing-related genes showed that splicing isoforms of Bcl-X, ICAD and ASF/SF2 were modulated in pnn-depleted cells. Minigene splicing assays indicated the pro-apoptotic form of Bcl-X (Bcl-Xs) and dominant-negative form of ICAD were found in pnn-depleted MCF-7 cells. Rescue of pnn-depleted MCF-7 cells with ASF/SF2 showed that apoptosis was arrested and altered alternative splicing events were reversed. With human umbilical vein endothelial cells (HUVEC), though Bcl-Xs is expressed in pnn-depleted cells, no apparent apoptotic event was observed. Conclusions: Pnn is essential for nuclear speckle assembly and for survival of cultured breast carcinoma cell line MCF-7 but not for normal HUVEC. The apoptosis triggered by pnn-depletion is through expression of pro-apoptotic Bcl-Xs and mediated by expression levels of ASF/SF2.

1238/B447

Functional Studies on SUMOylated Proteins Induced by Various Stresses employing Proteomic Strategies.

J. Yun, J. Jeong, J. Lee, K. Lee; College of Pharmacy and Division of Life & Pharmaceutical Sciences, Ewha Womans University, Seoul, South Korea

Post-translational modification by the reversible covalent conjugation of the small ubiquitin-related modifier (SUMO) peptide to specific lysine residues in the target protein is emerging as a major regular mechanism of protein function. Even though SUMO is the structurally related to ubiquitin, its biological functions are distinct to ubiquitin, yet poorly understood regulatory signal. In this study, we found that 293T cells overexpressing SUMO-1 are more protective to the cell death induced by various stresses including heat shock. This protection is caused from the increase of SUMOylation in response to stresses, which reduced the production of reactive oxygen species (ROS). To identify the sumoylated proteins in response to various stresses, we designed the mutants changing the C-terminal residues to determine the sumoylation target and sites. This mutant can conjugate to K residues of target proteins and can be cleaved by tryptic digestion for MS analysis. Several novel proteins were identified by amino acid sequencing using nano LC-ESI-q-TOF tandem MS employing this strategies. These results can provide the valuable information on the regulation mechanism of SUMO in stress response. [Supported by KOSEF NCRC for CCS & DDR. J Yun and J Lee supported by BK21]

Signal Transduction I (1239 – 1259)

1239/B448

Eukaryotic DNA Damage Response Is Coordinated by Ubiquitination of Two DNA Clamps.

W. Xiao, Y. Fu, Y. Zhu, K. Zhang; Microbiology and Immunology, University of Saskatchewan, Saskatoon, SK, Canada

In bacteria such as Escherichia coli, RecA is required for DNA damage tolerance through homologous recombination, replication restart and translesion synthesis to bypass replication blocks. In addition, the SOS response in E. coli is also initiated by RecA, which responds to DNA damage and facilitates the self-cleavage of the LexA repressor, resulting in increased expression of over 40 genes involved in cell survival under stress conditions. The latter phenomenon has not been previously described in eukaryotes, from yeast to human, and the involvement of eukaryotic RecA homologs (e.g., Rad51) in such a transcriptional regulation has been ruled out. In eukaryotic cells, a Rad6 (E2)-Rad18 (E3) ubiquitination complex is required for PCNA monoubiquitination after DNA damage, which lead to translesion synthesis and an error-free mode of bypassing replication blocks. We hypothesized that Rad6-Rad18 may serve to coordinate DNA damage response in eukaryotes. To test this hypothesis, we found that the Rad6-Rad18 ubiquitination complex is also required for the transcriptional response of a number of well-characterized genes in budding yeast and our microarray data indicate that deletion of RAD6 or RAD18 affects the expression of up to 380 damage-inducible genes. This function is independent of its monoubiquitination of PCNA, indicating an alternative target for Rad6-Rad18 ubiquitination. We fond that the Rad17 subunit of the PCNA-like 9-1-1 complex is subject to Rad6/Rad18- and DNA damage-dependent monoubiquitination and the Rad17-K197 residue with flanking sequences homologous to PCNA-K164, the Rad6-Rad18 substrate, is absolutely required to mediate the transcriptional response by Rad6-Rad18. Hence, the Rad6-Rad18 complex may be involved in the control of global gene regulation in a manner reminiscent of the bacterial SOS response. Furthermore, we demonstrated that Rad17 ubiquitination is also required for checkpoint functions possibly through damage-induced 9-1-1 clamp assembly and Rad53 phosphorylation. Based on these observations, we present a model in which Rad6 and Rad18 play key roles in coordinating DNA damage response through ubiquitination of two DNA clamps, PCNA and 9-1-1.

1240/B449

The Yeast GID Complex, a Novel Ubiquitin Ligase (E3) Involved in the Regulation of Carbohydrate Metabolism.

D. H. Wolf[1], O. Santt[1], T. Pfirrmann[2], B. Braun[1], J. Juretschke[1], P. Kimmig[5], H. Scheel[4], K. Hofmann[4], M. Thumm[3]; [1]Institut für Biochemie, Universität Stuttgart, Stuttgart, Germany, [2]University of Stockholm, Wenner-Gren Institute, Stockholm, Sweden, [3]Universität Göttingen, Institut für Biochemie und Molekulare Zellbiologie, Göttingen, Germany, [4]Miltenyi Biotec GmbH, Köln, Germany, [5]University of California, Department of Biochemistry and Biophysics, San Francisco, CA

Glucose-dependent regulation of carbon metabolism is central to cells. The switch from gluconeogenesis to glycolysis is associated with ubiquitin-proteasome linked elimination of the key enzyme fructose-1,6-bisphosphatase. Seven glucose induced degradation deficient (Gid)-proteins were found in a genomic screen and shown to form a complex that binds FBPase. One of the subunits, Gid2/Rmd5, contains a

degenerated RING finger domain. In an in vitro assay, heterologous expression of GST-Gid2 leads to polyubiquitination of proteins. A mutation in the degenerated RING domain of Gid2/Rmd5 abolishes fructose-1,6-bisphosphatase polyubiquitination and elimination in vivo. Six Gid proteins are present in gluconeogenic cells. A seventh protein, Gid4/Vid24, occurs upon glucose addition to gluconeogenic cells and is afterwards eliminated. Forcing abnormal expression of Gid4/Vid24 in gluconeogenic cells leads to fructose-1,6-bisphosphatase degradation. Gid4/Vid24 initiates fructose-1,6-bisphosphatase polyubiquitination by the GID complex and its subsequent elimination by the proteasome. Gid4/Vid24 acts as the central switch of this process. An additioal gluconeogenic enzyme, phosphoenolpyruvate carboxykinase, is also subject to GID complex-dependent degradation. The GID complex is a new type of ubiquitin ligase complex composed of novel subunits involved in carbohydrate metabolism. Gid4/Vid24 is a major regulator of this E3. An orthologous complex is found in mammalian cells.

1241/B450

A Proteomic Screen to Identify Substrates of the Ubiquitin-Proteasome System.

C. Y. Cheng, T. Chou, R. Deshaies; Biology, California Institute of Technology, Pasadena, CA

The ubiquitin proteasome system (UPS) is an important cellular mechanism responsible for the degradation of misfolded and unneeded proteins. By regulating levels of protein through proteolytic as well as non-proteolytic roles, the UPS helps control signaling pathways such as gene expression and the cell cycle. In the UPS, proteins are ubiquitinated via a series of covalent modifications by ubiquitin-conjugating enzymes. These ubiquitin-conjugated proteins are then delivered to the proteasome for degradation. However, the underlying mechanism of such selective delivery remains unknown. To facilitate the study of the UPS pathway, we first globally identified UPS substrates within the yeast proteome. We first obtained a Saccharomyces cerevisiae GFP fusion library of 4,159 different yeast strains, each containing a different GFP-tagged protein. In order to facilitate cellular uptake of bortezomib (a proteasome inhibitor) in our experiments, we disrupted the PDR5 locus, which codes for a drug efflux transporter, in each yeast strain. We analyzed our yeast strains in either 96-well or 384-well format, inoculating two wells per strain; one well contained control cells and the other contained bortezomib-treated cells. We quantified the steady state levels of each GFP fusion protein with and without proteasome inhibition by measuring the intensity of green fluorescence in a single cell resolution. We used fluorescence activated cell sorting (FACS) to identify 738 proteins that showed significant accumulation in Velcade treated cells. Using automated microscopy analysis, we found 816 potential UPS substrates. 274 proteins were identified as positives by both methods. We used Western blots to confirm the accumulation of 15 out of 24 proteins that had been identified as positives by both methods. The results from our FACS and microscopy screens suggest that approximately 300 UPS substrates may exist in yeast. Our research allows for future screens within the UPS proteome to identify substrates of specific UPS components, and further elucidate the UPS pathway.

1242/B451

Regulation of ROCKII Signaling Complexes.

S. Tumusiime[1], M. Rana[1], J. Guidry[1], V. Kurella[2], D. Worthylake[2], B. Worthylake[1]; [1]Pharmacology, Louisiana State University, New Orleans, LA, [2]Biochemistry, Louisiana State University, New Orleans, LA

Rho Kinase 2 (ROCKII) is a Serine/Threonine kinase that regulates multiple cell activities including cell adhesion, migration, invasion, cytokinesis and apoptosis. While the mechanisms that regulate ROCKII kinase activity are well-described, it is unclear how ROCKII signaling is specified temporally and spatially, to regulate different cell biological events. ROCKII has three domains: an N-terminal kinase domain, a regulatory Rho binding domain, and an uncharacterized C-terminal Pleckstrin Homology (PH) domain. PH domains bind phospholipids and also act as protein-protein interaction domains, both functions that may potentially influence the selection of downstream signaling partners. To identify candidate ROCKII signaling partners we used the ROCKII PH domain as bait in a proteomics-based screen. We identified a number of proteins associated with cytoskeletal dynamics, which was expected. However, we also found a significant number of proteins associated with membrane trafficking, leading us to predict that ROCKII localizes to membrane compartments within cells. Subcellular fractionation showed that ROCKII was enriched in both early endosomes and plasma membranes. Taken together, these data suggest a novel relationship between ROCKII and membrane dynamics. We then pursued confirmation of the interaction between ROCKII and two of the novel candidates identified in the screen for ROCKII signaling partners. Co-immunoprecipitation experiments were used to demonstrate that ROCKII forms a complex with Dynamin1 or Coronin1A in cells. Thus, we provide biochemical validation of our proteomics screen and have identified two novel ROCKII signaling partners. In conclusion, we hypothesize that the ROCKII PH domain regulates the composition and localization of multi-protein signaling complexes to determine which downstream signaling pathways are activated by ROCKII.

1243/B452

Comparison of Small GTPase Activation over Time after Growth Factor Stimulation in NIH 3T3 Cells.

S. Smith, R. Bomgarden, R. Farooqui, A. Deshpande, B. Kaboord; Thermo Fisher Scientific, Rockford, IL

Monomeric p21 GTP-binding proteins (small GTPases) serve as molecular switches in regulating a wide range of essential biochemical pathways in eukaryotic cells. GTPases cycle between active and inactive forms depending on whether GTP or GDP is bound, respectively. Small GTPases are typically grouped into five subfamilies: Ras, Rho, Rab, Ran and Arf, based on sequence homology and function. Here we analyzed the activation response for different members of the Ras, Rho, and Arf subfamilies including Ras, Rac1, Cdc42, Rap1, Rho, Arf1, Arf6, and RalA. Using GST fusions of specific GTPase downstream effectors, we selectively pulled-down active small GTPases after platelet-derived growth factor (PDGF) stimulation of serum-starved 3T3 cells. We also compared small GTPase activation over time with phospho-specific Western blots of proteins involved in PDGF signal transduction including PDGF receptor, Akt, MAPK, and Cdk1. Our results show that three Ras family members, Ras, Rap1 and RalA, are rapidly activated after PDGF receptor stimulation and phosphorylation. In addition, Rho, Rac1 and Cdc42 were also activated within the first hour after growth factor stimulation. In contrast, Arf1 and Arf6 showed maximum activation at times longer than four hours post PDGF stimulation. By using a global approach to observe cellular signaling transduction networks, we have gained insight into the interplay between small GTPase and kinase networks.

1244/B453

Scanning Mutagenesis of Gα12 Reveals a Role for the C-Terminal Region in RhoGEF Interaction.

B. J. Ritchie, L. A. Foster, B. R. Smith, T. E. Meigs; Biology, University of North Carolina - Asheville, Asheville, NC

The alpha subunit of the heterotrimeric G protein, G12, has been shown to modulate a variety of cellular processes including cell-to-cell adhesion, cell growth, and oncogenic transformation. Among proteins demonstrated as downstream effectors of G12 signaling, the Rho-specific guanine nucleotide exchange factors (RhoGEFs) p115RhoGEF and leukemia-associated RhoGEF (LARG) are of keen interest due to their demonstrated ability to activate the monomeric G protein Rho, leading to a diverse array of responses that include cytoskeletal rearrangements, cell migration, and transcriptional activation through serum response factor (SRF). The functional interaction between G12 proteins and RhoGEFs has been studied extensively using purified proteins; however, these studies have required the use of chimeric Gα proteins to obtain sufficient protein yields. In the present study, we have examined the interaction between a panel of engineered Gα12 mutants and immobilized forms of p115RhoGEF and LARG. We have identified several Gα12 mutants that are impaired in binding both RhoGEF proteins. Surprisingly, several mutations near the C-terminus of Gα12 disrupt interaction with these effector proteins. In conclusion, our findings implicate a previously unexamined region of Gα12 as critical for its interaction with RhoGEFs.

1245/B454

PDGF and FGF Receptors Access the Ras/Erk Signaling Pathway With Differential Inputs Through PI3K.

M. Cirit, C. Wang, J. Haugh; Chemical & Biomolecular Engineering, North Carolina State University, Raleigh, NC

Although it is appreciated that canonical signal transduction pathways represent dominant modes of regulation embedded in larger interaction networks, relatively little has been done to quantify pathway crosstalk in such networks. Through quantitative measurements that systematically canvas an array of stimulation and molecular perturbation conditions, together with computational modeling and analysis, we have previously elucidated crosstalk mechanisms in the platelet-derived growth factor (PDGF) receptor signaling network, in which phosphoinositide 3-kinase (PI3K) and Ras/Erk pathways are prominently activated. We have shown that, while PI3K signaling is insulated from crosstalk, PI3K enhances Erk activation in multiple ways. Whereas simultaneously blocking Ras and PI3K abolishes PDGF-stimulated Erk phosphorylation, each pathway makes an independent contribution to Erk activation, and PI3K affects Ras activation as well. The magnitudes of these effects depend strongly on the stimulation conditions, subject to saturation effects in the respective pathways and negative feedback loops. These results have now been extended to the signaling network utilized in response to FGF-2 stimulation of the same cell line. We show that the PI3K pathway is activated minimally in this context and, accordingly, Erk activation is essentially Ras-dependent. This system affords us the opportunity to quantitatively compare feedback regulation of signaling networks across receptor tyrosine kinases.

1246/B455

Prostaglandin A2 Induces the Expression of Heme Oxygenase-1 via ROS-P38mapk-Nrf-2 Pathway Requiring De Novo Protein Synthesis in U2OS Cells.

K. Ko, S. L, S. Jeong, H. Kim; Department of Biochemistry, College of Medicine, The Catholic University of Korea, Seoul, South Korea

Prostaglandin A2(PGA2) has been reported to induce expression of heme oxygenase(HO)-1 but the mechanism involved was not elucidated yet. In this work, we analyzed the signal transduction pathway leading to elevation of HO-1 expression. When U2OS (human osteosarcoma) cells were treated with PGA2, the expression of HO-1 increased at mRNA and protein levels. Accompanied with the expression of HO-1, accumulation of reactive oxygen species (ROS) and phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) and Erk1/2 were induced in PGA2-treated cells. PGA2 also augmented the protein level of p38MAPK. PGA2-induced HO-1 expression was almost completely prevented by pretreatment of N-acetyl cysteine (NAC, a thiol antioxidant), SB203580 (an inhibitor of p38MAPK), and cycloheximide (CHX) but not Erk1/2 inhibitors like PD98059 and U0126. In addition, CHX and NAC diminished the phosphorylation of p38MAPK and CHX also diminished the protein level of p38MAPK. Moreover, the induction of HO-1 was almost completely blocked in NF-E2-related factor 2 (Nrf-2)-knocked down cells. By immunocytochemical and immunoblot analyses, it was turned out that Nrf-2 is constantly present in nuclei and its expression was not changed by PGA2, CHX or SB203580. Taken together these findings suggest the followings: 1. PGA2 induces the expression of HO-1 via ROS-p38MAPK activation-Nrf-2 activation pathway. 2. P38MAPK activation by PGA2 may be achieved by phosphorylation and up-regulation of p38 expression. 3. Nrf-2 accumulation in nuclei is not sufficient for expression of HO-1 but its modification possibly by p38MAPK is critical for the transcriptional activity.

1247/B456

Role of p38MAPK and Heat Shock Proteins, HSP27 and HSP70, in Osmotic Stress in Renal vs. Blood Cells: A Comparative Study.

P. Patel, H. Tyagi, A. Khanna, M. Kasschau, S. Murphy; Biology, University of Sciences in Philadelphia, Philadelphia, PA

It has been observed that osmotic stress induces various changes in different cell types. Mammalian kidney cells are normally exposed to high osmotic concentrations as a consequence of their participation in the urinary concentration mechanism (Natalia et al, 2005). Cell structure and volume dependent remodeling of cytoskeleton is known to be influenced as an adaptive response to withstand high osmotic stress (Di Ciano et al, 2002). Using western blotting, we have investigated the expression of HSPs and p38MAPK in osmotic stress on hematopoietic KG1a cells, which on exposure to osmotic stress, exhibit filopodia formation. 35% reduction was observed in filopodial formation on being subjected to SB203580, a p38MAPK specific inhibitor. Similarly, a 75% decrease in filopodia formation was observed when KG1a cells were subjected to KNK-437 (HSP70 inhibitor) during osmotic stress (840 mOsm). This data along with the western analyses imply a role of HSP70 and p38 MAP kinase in osmotically induced signal transduction in KG1a cells. Using these inhibitors, we studied the expression of HSPs in kidney cells by western analyses. Studies with mIMCD-3 cells, the inner medullary collecting duct cell line and M-1, the cortical cell line from mouse, indicate that cells from different parts of the kidney respond differently to high osmotic stress. By western analyses we have observed over a 100% increase above control levels in the expression of HSP27 in M-1 and mIMCD-3 cells. Our studies show that kidney cells have higher basal expression of HSP70 than KG1a cells even under isotonic conditions. On being subjected to

hypertonic stress, KG1a cells exhibit a greater increase in HSP70 expression than kidney cells. It is possible that kidney cells constitutively express HSPs at high level to combat the stress. Results from western analyses are being confirmed by real time PCR.

1248/B457

Induction of Endothelial Nitric Oxide Synthase (eNOS) and Mitogen-Activated Protein Kinase (MAPK) Phosphorylation in Endothelial Cells by TP508.

S. A. Wadekar, D. N. Derkach, J. Cheung-Flynn, K. Perkins, M. Sheller; Research & Development, OrthoLogic Corp, Tempe, AZ

TP508, a 23-amino acid synthetic peptide representing residues 508 to 530 of human prothrombin, promotes eNOS activation and nitric oxide production in cultured coronary endothelial cells. In the ischemic heart, TP508 treatment results in endothelium-dependant vasodilation and reversal of endothelial dysfunction. The objective of this study was to characterize the cellular mechanism underlying the therapeutic effect of TP508. Cultured Human Aortic Endothelial (HAE) cells were treated with 150 µg/ml TP508 or 2U/ml thrombin for a period of 5 minutes, 30 minutes, 60 minutes and 24 hours. Cell extracts were resolved by SDS polyacrylamide gel electrophoresis. Phosphorylation of eNOS at Serine 1177 and MAPK at Threonine 202/Tyrosine 204 was detected by Western blotting. Within 5 minutes of treatment, thrombin elicited a three-fold induction of phosphorylated eNOS and a five-fold induction of phosphorylated MAPK. However, the effect of thrombin treatment diminished after 5 minutes and returned to baseline levels at later time points. Conversely, TP508 treatment exhibited a lower yet sustained 1.5-fold induction of phosphorylated eNOS and a two-fold induction of phosphorylated MAPK over the entire 24 hour period. In summary, thrombin and TP508 regulate eNOS- and MAPK-phosphorylation following distinct time courses of activation.

1249/B458

Development of MILLIPLEX™ MAP Immunoassays for the Analysis of MAPK/SAPK Cell Signaling Pathway Members.

F. Wiese, S. Harbison; Millipore, St Charles, MO

MAPK/SAPK signaling pathways play a role in the majority of cellular signaling events. The ability to monitor the concentration and phosphorylation status of MAPK/SAPK family member proteins is necessary for a thorough understanding of the physiological as well as pathological processes these pathways affect. To facilitate the analysis of MAPK/SAPK pathway members, we have designed a set of single-plex and multiplex Luminex® xMAP-based immunoassays. Assays were developed to analyze the MAPK/SAPK proteins: total and phospho p53 (pS15 and pS46), total and phospho ATF2 (pT69/71) and total and phospho STAT1 (pY701). Assay conditions were further optimized for the construction of a multiplex assay designed for the simultaneous analysis of the phosphorylated forms of ERK (pT185/pY187), JNK (pT183/pY185), p38 (pT180/pY182), HSP27 (pS78), c-jun (pS73), p53 (pS15), STAT1 (pY701), ATF2 (pT69/71), MSK1 (pS376) and MEK1 (pS218). Presented here are the results of several experiments designed to fully characterize these immunoassays using the HeLa human cell line stimulated by heat shock and arsenite treatment or the A431 human cell line treated with Epidermal Growth Factor. Assay response for both stimulated and non-stimulated cellular lysates was determined to be both specific and analyte concentration dependant. Immunoprecipitation combined with immunoblotting experiments corroborated the results observed for the developed immunoassays. These results demonstrate that the MAPK/SAPK analysis tools we have designed are an effective and efficient method to monitor critical constituents of these highly relevant cell signaling pathways.

1250/B459

Cdo Promotes Neuronal Differentiation via Activation of the p38MAPK Pathway.

H. Lee[1], J. Oh[1], G. Bae[1,2], Y. Yang[1], M. Yi[1], B. Kim[1], R. S. Krauss[2], J. Kang[1]; [1]Molecular Cell Biology, Sungkyunkwan University, Suwon, South Korea, [2]Developmental and Regenerative Biology, Mount Sinai School of Medicine, New York, NY

Neural basic helix-loop-helix transcription factors (bHLHs) control many aspects of neurogenesis, such as proliferation, fate determination and differentiation. We have previously shown that the promyogenic cell surface receptor Cdo modulates the Cdc42 and p38MAPK pathway via a direct association with two scaffold-type proteins, JLP and Bnip-2 to regulate activities of myogenic bHLH factors and myogenic differentiaion. We report here that Cdo employs similar regulatory mechanisms to promote neuronal differentiation. Expression of JLP a scaffold protein for p38MAPK, and Bnip-2, a regulator of Cdc42, is increased during differentiation of C17.2 neural precursor cells and P19 embryonal carcinoma cells. These molecules regulate Cdc42 and p38MAPK activities, which increase in a Cdo-dependent manner during neuronal differentiation of C17.2 cells and retinoic acidtreated P19 cells. Furthermore, enhancement or reduction of Cdc42 and p38MAPK activities enhances or reduces, respectively, neuronal differentiation of these cell lines. Cdc42 and p38MAPK activities also promote heterodimerization of Neurogenin1 and E47, suggesting that one way they promote neurogenesis is via regulation of neural bHLH factor activities. These results imply that a conserved intracellular signaling mechanism initiated by Cdo regulates the activities of tissue-specific bHLH factors and therefore functions as a key regulator of differentiation of several different cell lineages.

1251/B460

Phosphoproteomic Profiling of ERK MAP Kinase Signaling Reveals a Role of Phosphorylation in the Interaction of Nucleoporins with Transport Factors.

H. Kosako[1,2], N. Yamaguchi[2,3], C. Aranami[4], S. Kose[5], N. Imamoto[5], H. Taniguchi[1], E. Nishida[6], S. Hattori[2,4]; [1]Inst. for Enzyme Research, The University of Tokushima, Tokushima, Japan, [2]Inst. of Medical Science, The University of Tokyo, Tokyo, Japan, [3]BML, Inc., Saitama, Japan, [4]Sch. of Pharmaceutical Sciences, Kitasato University, Tokyo, Japan, [5]Advanced Science Inst., RIKEN, Saitama, Japan, [6]Grad. Sch. of Biostudies, Kyoto University, Kyoto, Japan

Many ERK MAP kinase substrates have been identified to date, but the diversity of ERK-mediated processes suggests the existence of additional targets. Here we present a phosphoproteomic approach to identify putative ERK substrates by combining the steroid receptor fusion system, IMAC, 2D-DIGE, and phosphomotif-specific antibodies. Purification of phosphoproteins from whole cell lysates by IMAC enabled sensitive detection of minor phosphorylated signaling components that would otherwise be obscured by abundant cellular proteins. Changes in phosphoprotein profiles between selective activation and inhibition of the Raf-MEK-ERK pathway were globally analyzed by 2D-DIGE. Quantitative analysis detected 37 reproducibly changed spots between ERK-activated and -inhibited samples, several of which

were recognized by the ERK consensus motif-specific antibodies. Mass spectrometric analysis identified 38 proteins including MEK1/2, ERK1/2, and previously known ERK substrates, demonstrating the feasibility of our approach. The remaining 24 proteins were considered novel candidates for ERK targets, which suggests as yet undefined roles for this signaling pathway in various physiological processes. ERK directly phosphorylated at least 13 proteins *in vitro*. Of these, the nucleoporin Nup50/Npap60 was verified as an *in vivo* ERK substrate by generating phospho-specific antibodies. Nup50 contains an N-terminal domain that binds to importin-α, a central FG repeat domain that binds to importin-β, and a C-terminal Ran binding domain. Interestingly, ERK phosphorylation of the FG repeat region of Nup50 was found to reduce its affinity for importin-β family proteins, importin-β and transportin. Other nucleoporins such as Nup153 and Nup214 also showed a similar functional change after ERK-mediated phosphorylation. Furthermore, energy-independent nuclear migration of importin-β and transportin was impaired in ERK-activated, digitonin-permeabilized cells. We propose that ERK may phosphorylate various nucleoporins to regulate nucleocytoplasmic transport. Our approach may be useful for large-scale identification of cellular kinase substrates.

1252/B461

Mechanism of Angiotensin II-Induced ERK1/2 Activation in Primary Fetal Cardiomyocytes.

X. Yin[1], M. Zhang[2], L. Hu[1], K. Catt[1]; [1]ERRB/NICHD, NIH, Bethesda, MD, [2]Departments of Medicine, Vanderbilt University School of Medicine, Nashville, TN

Transplantation of fetal cardiomyocytes has been utilized for studies on myocardial repair in the damaged hearts of rodents and other species. Cardiomyocyte growth and function are regulated by several G protein-coupled receptors, which exert their actions by activating intracellular signaling molecules such as p42/44 MAPK (ERK1/2). Angiotensin II (Ang II) is a potent mediator in the cardiovascular system, acting via its type 1 (AT1) and type 2 (AT2) receptors. However, the mechanisms by which Ang II receptors regulate ERK1/2 activation in fetal cardiomyoctes are not well-defined. We investigated the efficacy and specificity of Ang II for ERK1/2 activation in primary fetal cardiomyocytes by utilizing reverse transcription-polymerase chain reaction (RT-PCR), immunoblot, confocal fluorescence microscopy and small interfering RNA (siRNA) transfection. This revealed that activation of AT1 and AT2 receptors expressed in primary fetal cardiomyocytes positively regulates Ang II-induced ERK1/2 activation via Gq. Also, that Ang II-induced ERK1/2 activation is mediated by tyrosine kinases such as Src and the EGFR, but not by intracellular calcium. Interestingly, reduction of phospholipase C (PLC) and protein kinase Cα (PKCα) markedly increased ERK1/2 activation through c-Raf, and AT1R-mediated protein kinase A (PKA) activation negatively regulated ERK1/2 activation by other signaling pathways in fetal cardiomyocytes stimulated by Ang II. Our observations that PLC, PKCα and PKA mediate the feedback inhibition of ERK1/2 activation provide new insights into the complex interactions between MAPK activation and other signaling pathways in Ang II receptor signaling. These findings reveal that the mechanism of Ang II-induced ERK1/2 activation has distinct features in fetal cardiomyocytes, and that expression of the AT2 receptor could be relevant to the physiological functions of cardiomyocytes, such as differentiation, proliferation and survival. The observed mechanism of ERK1/2 activation via the synergy of both AT1 and AT2 receptors could be relevant to clinical approaches using cell-based therapy for diseases such as heart failure and cardiac hypertrophy.

1253/B462

A Functional Sirna Screen Identifies Novel Regulators of mTORC1 Signaling.

G. Hoffman[1], N. Moerke[2], M. Hsia[1], C. Shamu[2], J. Blenis[1]; [1]Cell Biology, Harvard Medical School, Boston, MA, [2]Institute of Chemsitry and Chemical Biology, Harvard Medical School, Boston, MA

Several fundamental aspects of the signaling network responsible for mTORC1 activation remain poorly understood. Specifically, although the role of TSC2 in down regulating Rheb following growth factor activation is well established, neither the mechanisms responsible for up-regulating the pathway by stimulating Rheb GTP-binding, nor the mechanism by which Rheb stimulates mTORC1 kinase activity, have been well characterized. In addition, the mechanism by which mTORC1 kinase activity is regulated by cellular amino acid levels is poorly defined; the upstream amino acid sensor has not been identified nor is it apparent how amino acid depletion impinges on mTORC1 activity. In order to address these questions, we developed a high throughput, cell-based assay for measuring phosphorylation of rpS6 at Ser235/236 in mammalian tissue culture cells. This assay takes advantage of the 'In Cell Western' (ICW) technique using the Aerius infrared imaging system (LiCOR Biosciences). Using this approach, we have preformed a large scale siRNA screen in human cancer cells to identify likely targets for pharmacological inhibition of the pathway. As both growth factor and amino acid inputs are required for full activation of mTORC1 and subsequent phosphorylation of rpS6, knockdown of components required for both the growth factor and amino acid branches of the mTORC1 signaling network lead to a reduction in the phospho-rpS6 signal and score as hits in the primary screen. Importantly, known kinases involved in growth factor-mediated inputs leading to rpS6-phosphorylation, including IRS2, IGF1R, PI3-kinase, PDK1, and S6K2, score as hits in the screen, validating our approach. In addition to these known pathway components, a number of uncharacterized genes scored as strong hits in our screen and we are currently characterizing their mechanism of action. Bioinformatics analysis of the siRNA data set identifies a number of conserved pathways that play previously unappreciated roles in regulation of mTORC1 activity which may serve as novel targets for therapeutic intervention for the treatment of cancer and metabolic diseases associated with deregulated mTORC1 signaling.

1254/B463

Ras/Raf/Mek/Erk Signal Attenuation by the PHLPP Phosphatases.

M. Niederst[1], G. Reyes[2], M. Kunkel[2], J. Enserink[3], A. Newton[2]; [1]Biomedical Sciences Graduate Program, UCSD, La Jolla, CA, [2]Pharmacology, UCSD, La Jolla, CA, [3]Cell and Molecular Medicine, UCSD, La Jolla, CA

The recently discovered PH Domain Leu-Rich Repeat Protein Phosphatase (PHLPP) family of proteins was found in a search to understand how Akt signaling is terminated (Gao et al., Mol Cell 18 p.13, 2005; Brognard et al., Mol Cell 25 p.917, 2007). The two isoforms PHLPP1 and PHLPP2 control the amplitude of agonist-induced signaling by directly dephosphorylating and inactivating Akt, thus suppressing cell proliferation and inducing apoptosis. Given the dramatic effect of PHLPP in antagonizing cell proliferation, we asked whether PHLPP plays a role in regulating additional proliferative signaling pathways, specifically the Ras/Raf/Mek/Erk pathway. Data from proliferation assays indicate that PHLPP suppression of the cell cycle is mediated by down-regulation of both Akt and Erk pathway signaling. siRNA

experiments confirmed that loss of PHLPP1 or PHLPP2 in normal breast cells, as well as breast cancer and glioblastoma cells, leads to increased phosphorylation of Mek and Erk following EGF stimulation compared to vector controls. Kinetic analyses indicate that both the amplitude and duration of Erk and Mek phosphorylation increases following EGF stimulation in cells lacking PHLPP1 and PHLPP2. Investigation of upstream pathway components showed that PHLPP1/2 also suppress Ras activation following agonist stimulation. Finally, Western blot and imaging analysis revealed that PHLPP1/2 negatively regulate EGFR protein levels in normal and cancer cells, and consistent with this, negatively regulate the collective signaling of EGFR in response to EGF. Thus, PHLPP isoforms control signal termination of two critical oncogenic pathways that are often misregulated in cancer, the Akt and Erk pathways. Furthermore, PHLPP1/2 act to reduce the levels of EGFR, a gene whose levels are upregulated in a large percentage of human cancers. Taken together, these results strengthen the case for PHLPP1/2 as putative tumor suppressor proteins.

1255/B464

Changes in the MAP kinase Pathway Lead to Altered Response to Induced Oxidative Stress upon Contact Inhibition.

M. Gaballah, M. Slisz, D. Hutter; Biology, Monmouth University, West Long Branch, NJ

Cells in different stages of proliferation demonstrate changing levels of MAP kinase pathway proteins, which may affect the response of cells in culture to oxidative stress. Previous results indicate increased MAP kinase phosphatase (MKP) levels upon contact inhibition in healthy fibroblasts, corresponding with decreased levels of phosphorylated extracellular signal-regulated kinase (ERK) and p38. Cancerous fibrosarcoma cells do not exhibit contact inhibition, and no change to active kinase or MKP levels was seen. Therefore, a relationship must exist between proliferation and MAP kinase and phosphatase activity, and decreased proliferation due to overexpression of MKP-1. JNK-1, another downstream target protein, remained inactive in cells during all stages of proliferation. However, following induced oxidative stress by H2O2, subconfluent cells exhibited different responses than those in the confluent stage. Although phosphorylated JNK (p-JNK) was seen in both stages of growth, higher levels of p-JNK were detected in subconfluent cells, along with cleaved PARP, indicating the early stages of apoptosis. Therefore, a correlation between confluency and response to oxidative stress may occur.

1256/B465

Development of a Highly Sensitive Assay for High Throughput Detection of Cyclic AMP

M. Slater; Millipore, Temecula, CA

cAMP is a key second messenger in signal transduction, and an important modulator of metabolism, cancer, inflammation and CNS signaling. Agents which increase or decrease intracellular cAMP are of major interest in basic research and drug discovery. Our aim was to develop a highly sensitive competitive immunoassay for rapid and ultra-sensitive quantitation of cAMP, and to validate the assay for GPCR screening applications. We hypothesized that by using a highly sensitive and specific anti-cAMP antibody, and an optimized alkaline-phosphatase labeled cAMP tracer, it may be possible to both obtain high levels of sensitivity and reduce the incubation times necessary in this type of assay. By careful selection of the cAMP tracer and AP substrate, and by optimizing the dilutions of anti-cAMP antibody and cAMP tracer, we were able to deliver reproducible results in an assay lasting only 60-90 minutes. We present data showing that the assay is highly sensitive, reproducible and suitable for use in drug discovery applications. The dynamic range of the assay was determined to be 10^-1 - 10^5 pmol/mL (6 log dynamic range). Sensitivity was determined as 0.034 pmol/mL in acetylated samples and 0.26 pmol/mL in non-acetylated samples. We also demonstrate that the assay shows excellent linearity and precision, and cross-reactivity of the anti-cAMP antibody with other nucleotides is extremely low (<0.1%). We present data indicating the utility of the assay for GPCR screening applications. In CHO(wt) cells seeded at 10,000 cells per well, forskolin dose-dependently increased cAMP (EC50 3.5 µM). In another experiment CHO cells transfected with a Gs-coupled GLP-1 receptor were cloned, a dose response range of several putative ligands was added, and EC50 values for cAMP production determined. Additionally, the colorimetric signal developed by the assay was extremely stable, with identical EC50 values being calculated up to 24 hrs after completion of the experiment. This property of the assay greatly facilitates large-scale screening. In summary, the assay we present is sensitive, highly specific for cAMP, and may be used to rapidly test a wide variety of sample types from all species.

1257/B466

Ras Is Required for cAMP-dependent Rap1 Activation via Epac2.

C. Liu[1,2], M. Takahashi[1], Y. Li[1], S. Song[3], P. J. Stork[1,2]; [1]Vollum Institute, Portland, OR, [2]Cell & Developmental Biology, Oregon Health & Science University, Portland, OR, [3]School of Optometry, University of California, Berkeley, Berkeley, CA

Exchange proteins activated by cAMP 2 (Epac2) is a guanine nucleotide exchange factor (GEF) for Rap1, a small G protein involved in many cellular functions including cell adhesion, differentiation and exocytosis. Epac2 is thought to be selectively activated by cAMP, yet whether other molecules regulate Epac2 function is unknown. Epac2 also interacts with Ras-GTP via a Ras association (RA) domain, but previous studies suggested that the RA domain was dispensable for Epac2 function. Here, we show for the first time that Ras and cAMP regulate Epac2 function in a parallel fashion, and the Ras-Epac2 interaction is required for the cAMP-dependent activation of endogenous Rap1 by Epac2. The mechanism for this requirement is not allosteric activation of Epac2 by Ras, but the compartmentalization of Epac2 on the Ras-containing membranes. A computational modeling reveals that this compartmentalization is a function of both the level of Ras activation and the affinity between Ras and Epac2. In PC12 cells, a well established model for sympathetic neurons, the Epac2 signaling is coupled to activation of mitogen-activated protein (MAP) kinases and contributes to neurite outgrowth. Taken together, Epac2 is not only a cAMP sensor but also a bona fide Ras effector. Coincident detection of both cAMP and Ras signals is essential for Epac2 to activate Rap1 in a temporally and spatially controlled manner.

1258/B467

Differential Regulation of Myocardial Collagen Formation by Adenylyl Cyclase Subtypes in Cardiac Hypertrophy.

H. Qiu, L. Yan, L. F. Fritzky, C. Hong, S. Gao, M. Park, A. Guellich, Y. Ishikawa, K. Iwatsubo, J. Sadoshima, D. Vatner, S. F. Vatner; Cell Biology and Molecular Medicine, UMDNJ, New Jersey Medical School, Newark, NJ

Adenylyl cyclase type 5, when it is knocked out (AC5 KO), and type 6, when it is overexpressed transgenically (AC6 Tg) have been reported to protect the heart in response to stress. If so, AC5 Tg and AC6 Tg should exert opposite effects, and microarrays of their gene profiles should provide insight into mechanisms mediating the opposing actions. Accordingly, RNA was extracted from the hearts of AC5 and AC6 transgenic mice (Tg) and wild type (WT) mice (n=5/group) and hybridized to mouse microarrays (Gene Chip 430.2.0, Affymetrix, Santa Clara, CA). The results indicated that collagen genes, including type I, III, V and VI, were significantly downregulated in AC6 Tg compared with WT. Our hypothesis is that the AC6 Tg, compared with AC5 Tg, would be protected in response to chronic pressure overload, and that the effects on collagen and fibrosis would be opposite in these models. Both AC5 and AC6 mice were subjected to aortic banding for 1 week and compared with WT. After 1 week banding, left ventricular (LV) function, as reflected by LV ejection fraction (LVEF), was reduced ($P<0.05$) in AC5 Tg (56±4%) compared with WT (77±1%), but not in AC6 Tg (73±2%) or in AC5 KO (72±3%). Collagen content was quantitated with picric acid sirius red (PASR) staining and was significantly higher ($P<0.05$) in AC5 Tg (1.52±0.14) than in AC6 Tg (0.83±0.2) after banding. Collagen content in AC5 KO was similar to AC6 Tg after aortic banding (0.65±0.1) and also significantly less, $P<0.05$, than AC5 Tg, suggesting an anti-fibrotic role for AC6 and for AC5 KO in the adult mouse heart during cardiac remodeling. In addition, western blotting demonstrated paradoxical increases in AC5 protein, but decreases in AC6 protein following aortic banding, which would tend to enhance the differences in collagen in the Tg models, but not in AC5 KO, which is devoid of AC5. Therefore we conclude that there is a major difference in regulation of collagen in AC5 Tg vs AC6 Tg and AC5 KO mice in the cardiac remodeling following pressure overload, coupled with opposite responses of AC5 and AC6 protein with aortic banding, which may explain the diametrically opposite responses to stress with cardiac overexpression of AC5 vs AC6, or vs AC5 KO.

1259/B468

Dissection of Gα12-mediated Signaling Pathways through a Mutational Analysis of Effector Binding.

E. S. Fisher, E. R. Montgomery, C. M. Olson, B. J. Ritchie, T. Y. Choi, T. E. Meigs; Biology, University of NC Asheville, Asheville, NC

The heterotrimeric G protein α-subunit Gα12 has been implicated in a variety of cellular responses, including signaling pathways leading to cancerous proliferation and invasion. Efforts to decipher the mechanism of Gα12 action have been complicated by the discovery of more than a dozen putative effector proteins that directly interact with Gα12. Our objective has been to dissect this signaling network using a protein-protein interaction screen, in which various downstream binding partners of Gα12 are engineered as fusions to glutathione-S-transferase (GST) and then assessed for binding to a comprehensive panel of amino acid substitution mutants within Gα12. Thus far, we have engineered and expressed GST-fusions of the Gα12 effector proteins E-cadherin, radixin, heat shock protein-90, protein phosphatase-5, axin, and several Rho-specific guanine nucleotide exchange factors. We have demonstrated each of these immobilized fusion proteins to precipitate epitope-tagged, constitutively active Gα12 from HEK293 cell lysates. Subsequently, our screening of these fusion proteins for precipitation of Gα12 mutants has revealed several 6-amino acid substitutions as selectively disrupting Gα12 interaction with a particular target protein. Of particular interest, we have identified two Gα12 mutants that fail to bind E-cadherin while retaining binding to the other effectors listed above. In conclusion, these results validate this approach as a method for unraveling the numerous signaling axes emanating from activated Gα12, and set the stage for future studies in which these "selectively uncoupled" mutants can be used to assess the cell biologic role of Gα12 interaction with either E-cadherin or other specific target proteins.

Bioinformatics/Biological Computing (1260 – 1269)

1260/B470

The Open Microscopy Environment: Informatics and Quantitative Analysis for Biological Microscopy.

J. Swedlow[1,3], C. Allan[1], C. Blackburn[1], J. Burel[1], M. Linkert[3,2], B. Loranger[1], D. Macdonald[1], J. Moore[3], W. J. Moore[1], A. Patterson[1], C. Rueden[2], A. Tarkowska[1], I. Goldberg[4], K. Eliceiri[2]; [1]Wellcome Trust Centre for Gene Regulation & Expression, University of Dundee, Dundee, United Kingdom, [2]Laboratory for Optical and Computational Instrumentation, University of Wisconsin, Madison, WI, [3]Glencoe Software, Inc, Seattle, WA, [4]Image Informatics and Computational Biology Unit, National Institute for Aging, Baltimore, MD

Many advances have been made in instrumentation for light microscopy and image analysis algorithms, but computational cell biology remains out of reach for many investigators. Proprietary file formats and closed, non-standardised programming interfaces make integration of new imaging methods and algorithms difficult at best. We have developed an open-source software framework to address these limitations called the Open Microscopy Environment (http://openmicroscopy.org). OME has three components—an open data model for biological imaging, standardised file formats and libraries for data file conversion and applications for image data management and analysis. The OME Data Model (http://ome-xml.org) is a foundation for the whole OME project, and has recently been updated to more fully support fluorescence filter sets, unique identifiers, and imaging using multi-well plates. Bio-formats (http://loci.wisc.edu/ome/formats.html) is a pluggable Java library for reading data from proprietary file formats and the OME-TIFF file format (http://loci.wisc.edu/ome/ome-tiff.html) is an open specification for an exchangeable image data file. These resources enable access to data by different processing and visualization applications, and data sharing between collaborators. The OMERO application platform combines an image metadata database, a binary image data repository and an image management application to provide a multi-user image management, visualization, and analysis facility. OMERO includes OMERO.server, a Java application running inside a JBOSS application server; a web-based OMERO.webadmin for user administration; OMERO.insight, a rich client for image data management, visualization, and analysis, and OMERO.importer, a client application that uses Bio-Formats for proprietary data file import into OMERO.server. The Beta3 release of OMERO (http://trac.openmicroscopy.org.uk/omero/wiki/MilestoneDownloads) includes interfaces for C/C++ and Python to support a range of client applications, a flexible, fully-indexed image tagging and annotation system and an interface into Matlab. Demos of OMERO (http://openmicroscopy.org/site/videos) and roadmaps are available (http://trac.openmicroscopy.org.uk/omero/roadmap).

1261/B471

Network-based Diagnosis and Modelling of Cancer Development and Progression.

H. Chuang[1,2], T. Ideker[1,2], E. Lee[3], D. Lee[3]; [1]Bioinformatics, Univ. of California San Diego, La Jolla, CA, [2]Bioengineering, Univ. of California San Diego, La Jolla, CA, [3]Bio and Brain Engineering, Korea Advanced Institute of Science and Technology, Daejeon, South Korea

The advent of microarray technology has made it possible to classify disease states based on gene expression profiles of patients. However, expression-alone classification can be challenging in complex diseases due to factors such as cellular heterogeneity within a tissue sample and genetic heterogeneity across patients. Moreover, it remains unclear how these marker genes interrelate within a larger functional network. A promising technique for coping with these challenges is to incorporate pathway information into the disease classification procedure in order to classify disease based on the activity of entire signaling pathways or protein complexes. We have proposed a novel approach to integrate gene expression with protein interactions to dissect breast cancer metastasis. Gene expression profiles from large cohorts of patients were mapped to a huge human protein interaction network. A search over this network was performed to identify discriminative subnetworks which could be used to classify the disease status of a patient. The new prognostic markers, not as individual genes but sets of co-functional genes, predict the risk of metastasis more accurately than previous approaches based only on gene expression. More than being more reproducible and robust, our subnetworks include more mutated genes which are excluded in the classical analyses and also give molecular models for how these genes might be associated with cancer metastasis. Currently we have been extending this new integrated analysis to unveil the mechanisms underlying other cancers, such as leukemia, prostate cancer and lung cancer. By incorporating canonical pathway knowledge, we identified condition-responsive genes within pathways to infer the level of pathway activation perturbed by disease status. As the promising protein subnetwork markers for predicting metastasis potential, our pathway markers also lead to better classification performance for diagnosis and prognosis of these various cancers. The novelty and broad interest of the work relate to its integration of network analysis with microarray classification. We are aware of few if any studies which use protein networks to improve classification accuracy.

1262/B472

From Crosstalk of Signaling Pathways to Cellular Fate Patterns.

C. Giurumescu[1], A. Asthagiri[2]; [1]Division of Biological Sciences, University of California San Diego, La Jolla, CA, [2]Division of Chemistry and Chemical Engineering, California Institute of Technology, Pasadena, CA

During development of an organism descendant cells of the fertilized egg interact via signaling pathways to induce the formation of the adult body form through specification of cell fates. Using the vulva development of C. elegans as a model system, we sought to understand how a biomolecular network ensures a robust spatial pattern of cell fates. Patterning in this system depends on a spatial gradient of a soluble factor (LIN-3), and on direct cell-cell contact (LIN-12). These two fate patterning signals are coupled intracellularly: lateral cell-cell interactions inhibit signaling mediated by the soluble factor; meanwhile, the soluble factor regulates the extent of lateral signaling. We formulated a mathematical model which shows that coupling these two extracellular signals amplifies the cellular perception of the LIN-3 gradient, polarizes lateral signaling, and enhances fate segregation beyond that of an uncoupled system. We conduct a broad sweep of parameter values in essence performing in silico genetics experiments. Using a phenotype mapping scheme, we show that in silico mutations lead to the same phenotypes as their experimental counterparts. Our model predicts the cryptic quantitative evolution of this coupled network inferred experimentally from different Caenorhabditis species. Since both LIN-3 and LIN-12 are conserved signaling molecules in worms, flies and mammals, our study provides new insight into mechanisms guiding development.

1263/B473

Bioinformatic Analysis of the Rat Genome.

J. Kazi, J. Soh; Department of Chemistry, Inha University, Incheon, South Korea

Protein kinases play crucial roles in regulation of signaling pathways. In order to identify protein kinases in the rat genome, publicly accessible genetic sequence databases were searched and analysed. These searches yielded a catalog of 555 protein kinases and 45 protein kinase pseudogenes. At least 509 human protein kinases have rat orthologs, confirming rat as a model system for studying human protein kinases. Rat has almost all protein kinases as orthologous pairs that present in the mouse genome. The comparison of 11 different eukaryotic species revealed the evolutionary conservation of protein kinases. Two hundred and seventy nine rat protein kinases contain 128 functional domains other than kinase domain and 268 rat protein kinases have a nuclear localization signal. Finally, a user-friendly web-based resource which consolidates the gene and protein annotations for all human, mouse and rat protein kinases has been developed.

1264/B474

A Data-mining Approach to Prioritize Candidate Binding Partners of Biogenesis of Lysosome-related Organelles Complex-1 (BLOC-1).

I. A. Rodriguez-Fernandez, V. T. Cheli, E. Dell'Angelica; Human Genetics, University of California Los Angeles (UCLA), Los Angeles, CA

Large-scale studies of protein-protein interaction, using mainly the yeast two-hybrid system, have been publicized as important contributions towards understanding protein function. Thousands of novel interactions obtained through this type of studies are available in various databases. However, following up experimentally such great amount of data can become overwhelming. In this work, we explored ways of efficiently gathering high-quality information about the candidate binding partners of a protein complex of interest, BLOC-1, in order to select the most relevant candidates for subsequent experimental work. BLOC-1 is a protein complex that comprises eight subunits, some of which (e.g., dysbindin) are encoded by genes that have been linked genetically to Hermansky-Pudlak syndrome and schizophrenia. More than a hundred candidate binding partners of BLOC-1 subunits were found through scientific literature and database searches, and ranked using specific criteria: presence of predicted coiled-coil-forming, transmembrane and functional domains, presence or absence of orthologs in selected species, reported function and any additional experimental evidence for the interaction. Candidates among those that ranked at the top, including Rab11 and some SNARE proteins, were tested experimentally for their biochemical interaction in the context of native BLOC-

1 and, in the case of Rab11, for its genetic interaction with components of the pathway for biogenesis of lysosome-related organelles in flies. This work demonstrates how relatively simple data-mining approaches can be used to prioritize candidate binding partner for further experimental characterization.

1265/B475

Functional Characterization of Arabidopsis Tubby Domains Using Computational Methods.

V. Nataraj Dongre, D. Brogun, S. M. Singh; Research Foundation, City University of New York, Brooklyn, NJ

Tubby domain containing proteins are cell signaling proteins common to many multicellular eukaryotes. The tubby domains have dual binding function and are capable of interacting with both DNA and phosphotidylinositol, thereby potentially functioning as transcription factors as well as membrane associated signaling factors. Structurally they adopt an unusual helix-filled β-barrel structure with a putative DNA binding groove that terminates in a phosphoinositide binding basic pocket as revealed in experimentally solved tubby domains. Tubby and tubby-like proteins have been implicated in the maintenance and function of neuronal cells during post differentiation and development, and mature-onset obesity in animals but not much is known about the function of tubby-domain proteins in plants. We have undertaken a comprehensive computational examination of all tubby domains in the model plant, Arabidopsis thaliana to understand the structural basis for the mechanism of their function and to compare them with tubby-domains in other organisms. We have modeled the various tubby-domain proteins in A. thaliana, which share 30- 80% sequence similarities across their C-terminal tubby domains and are unique in possessing a conserved N-terminal F-box domain of about 50 residues in almost all members, using an automated modeling pipeline coupled with manual refinement methods. The biophysical analysis of all tubby domains in this model plant represents the first structural investigation of these domains in plants and provides initial insight and predictions as to their function in plants.

1266/B476

Imitational Modeling of Cytoskeleton: Dynamics, Differentiation, Active Transport and Membrane Interactions.

A. Shpilman[1], E. Nadezhdina[2]; [1]Bioengeneering and Bioinformatics, Moscow Lomonosov's State University, Moscow, Russian Federation, [2]Cytoskeleton research, Intstitute of Protein Research, Puschino, Russian Federation

Imitational modeling is a new way to model dynamic intercellular dynamic structures such as cytoskeleton, active transport and cellular motility systems. It combines particular interactions with stochastic algorithms to acquire models with variatonal complexity and accuracy. Microtubules network has been "imitationally" modeled as system of chains of tubulin clusters. Clusters assemble into microtubules and exclude from them randomly according to probability coefficients. Microtubule behavior defined by 20 coefficients, including constants of association and dissociation at plus and minus ends of microtubule for different GTP hydrolysis state of tubulin. Modern desktop computers can coordinate a number of virtual tubulin molecules close to real one. Apart from that, differentiation of cellular space is provided by insertion different zones of modified constants, including microtubule-nucleating centrosome, anchoring area (hypothetically Golgi apparatus) and membrane borders. The resulting model reflects many features of microtubule systems in vivo, including geometry, dynamics and responses to number of experiments such as partial stabilization of microtubules with taxol. By changing various coefficients of the system, we can observe influence of centrosome properties or cellular margin on microtubule system, for better understanding of system dynamics and planning in vivo experiments. The next step is modeling of active transport by inserting dynein and dynactin transport molecules that connects to microtubule along with differentiated distribution of transported molecules concentration. These transport molecules act by stochastic rules in step by step manner. This approach combined with standard imitational modeling feature - visualization of whole process - gets us information about mechanisms and rates of localization of macromolecules in cellular space, such as stress granules assembly. Imitational modeling approach combined with quantum sources of true stochastic distributions will in future gives us opportunity to transfer real life structures in silico with maximum likelihood. Modeling of cytoskeleton due to its "imitational" nature is a great start.

1267/B477

Mathematical Modeling of Matrix-Dependent Endothelial Cell Networking in 4-D.

A. Klinger[1], C. Hurtubise[1], S. Sweeney[1], A. Lucia[1], P. Jones[1,3], J. E. Sabin[2]; [1]Institute for Medicine & Engineering, University of Pennsylvania, Philadelphia, PA, [2]Department of Architecture, University of Pennsylvania, Philadelphia, PA, [3]Department of Pathology & Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Networking of endothelial cells during development relies upon dynamic remodeling and subsequent stabilization of cell-cell and cell-extracellular matrix (ECM) interactions. Herein, we investigate lung endothelial cells network dynamics using thin films of engineered ECM, including type I collagen and a reconstituted basement membrane. We present methods for establishment of “D’Arcian” biological coordinates for these systems and compare the applicability of: (1) applied pattern growth theory with identification of emergent growth seeds and level sets and; (2) the cellular Potts model. We further describe convergent non-linear least-squares determination of attraction force field parameters and evolution, through standard Monte Carlo procedures, toward cell-cell and cell-matrix interaction energetic partitioning.

1268/B478

Percolation Thresholds for Diffusing Particles of Nonzero Radius: Circular Obstacles in the Two-Dimensional Continuum.

M. J. Saxton; Biochemistry & Molec Med, Univ of California, Davis, CA

Lateral diffusion in the plasma membrane is obstructed by proteins bound to the cytoskeleton, and the rates of diffusion-mediated reactions in the membrane may therefore depend on the effects of obstruction. The most important parameter describing diffusion in the presence of immobile obstacles is the percolation threshold. At obstacle concentrations above the percolation threshold, long-range connected paths disappear and the long-range diffusion coefficient goes to zero. The thresholds are well-known for point diffusing particles on various lattices or the continuum. But for particles of nonzero radius, the threshold depends on the excluded area, not just the obstacle concentration. Earlier results [Saxton, Biophys J 64 (1993) 1053] for the triangular lattice showed a very rapid decrease in the threshold as the radius of the

diffusing particle increases, but a lattice model gives very low resolution. The current work finds the percolation threshold for circular obstacles in the two-dimensional continuum as a function of the radius of the diffusing species. The thresholds are obtained by a Monte Carlo method based on the Voronoi diagram for the obstacles. Each Voronoi bond is by definition the path equidistant from the nearest pair of obstacles, so the separation of that pair determines whether a diffusing particle of a given diameter can traverse that bond. For the simplest case, point obstacles, one can therefore choose a threshold corresponding to the diameter of the diffusing particle, set the conductivity of all bonds narrower than that diameter to zero and all wider bonds to one, and test for bond percolation on the resulting Voronoi diagram. The results are used to find the thresholds for lipids and for proteins of different diameters. (Supported by NIH grant GM038133)

1269/B479

Genomic Feature Selection: Mixing Dynamics and De-Mixing Strategies.

E. Capobianco; CRS4 Bioinformatics, Pula, Italy

High-throughput microarray technologies measure the abundance of thousands of mRNA targets simultaneously. The typical problem linked to dimensionality is due to the disparity between available samples, say by limited conditions such as time course experiments, and gene expression changes from entire genomes. By pursuing reverse engineering in the attempt to reconstruct the gene-gene interaction network from experimentally observed expression change measurements, the goal is to identify network structure, such as hubs, and dynamical features, such as regulatory paths, for then quantifying the strength of gene connectivity map with some confidence. This problem is inverse in its nature, and as such it refers to challenging statistical inference aspects. One should start by defining model selection strategies, then setting regularization approaches, and finally developing algorithms to deal with the high dimensionality and perform efficiently at the scale of N-body computations. From the standpoint of statistical accuracy of results, the issue of dealing with extremely finite-sized data is crucial. To this end, existing experimental replicates should be exploited, from both a biological and a technical side. How crucial is looking at replicates is indeed what we aim to show for the study at hand. In particular, feature selection is performed with projective methods, and the quality of results provided depends on the information content of the available replicates. Through a quite simple and heuristic sieving strategy, the issue of the appropriateness of averaging information among replicates is questioned. Then, the problem of mapping the observed high-dimensional variables to a low dimensional feature space is studied. The conclusions we support with our data suggest that the extracted gene features can be stabilized with respect to the inherent variability of expression values measured in triplicates, which in turn appears to lead to a better inference of the underlying gene regulatory maps.

Molecular Biology (1271 – 1290)

1271/B481

RNA Quality Assessment and the Effect on Gene Expression Studies.

B. Lam[1], V. Van Deventer[1], C. Dobbin[1], Y. Haj-Ahmad[2,1], N. Rghei[1], P. Roberts[1]; [1]Norgen Biotek Corp., Thorold, ON, Canada, [2]Brock University, St. Catharines, ON, Canada

Gene expression analysis using RNA has played a central role in our understanding of many aspects of the biology of an organism. In the past decade a number of novel gene expression technologies were developed, including Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR) and various microarray platforms. The quality of the starting RNA material is important in obtaining meaningful gene expression data. Traditionally, researchers used optical density (OD) and agarose gel electrophoresis to assess the quality and integrity of RNA. Recently, RNA quality is being evaluated by a capillary electrophoresis-based Bioanalyzer. RNA Integrity Number (RIN), based on the shape of the resolution of the RNA in the Bioanalyzer, has become a common parameter in RNA quality control. Interestingly, RNA isolated using popular methods such as commercial guanidine/phenol-based extraction do not resolve well in the Bioanalyzer but work properly in most downstream applications. Here we compared various RNA quality parameters and their correlation with RNA performance in downstream expression studies. RNA, including small RNA or miRNA, was isolated from tissue, plasma or cultured cells using three commercial methods: classic phenol-based isolation, phenol-extraction followed by silica-based column cleanup, and novel silicon carbide column-based extraction without the use of phenol. The quality of RNA extracted was evaluated with OD, agarose gel electrophoresis and the Agilent 2100 Bioanalyzer. RT-qPCR was performed to amplify different transcripts including large and small housekeeping mRNA, as well as miRNA. While the quality of RNA isolated by all methods was similar according to OD and agarose gel visualization, there was a large variation in RIN values from the BioAnalyzer. In particular, some of the isolations involving phenol extraction, including those with column cleanup, obtained RIN less than 3, while they resolved well on agarose gels. In addition, no inhibition of RT-qPCR was observed from all samples isolated from HeLa, both for regular mRNA and miRNA. Based on these data, we proposed a new guideline for RNA quality assessment for gene expression studies.

1272/B482

Understanding the Function of MTGR1a Protein Using Protein Protein Interaction and Microarray.

L. Vig, V. Kumar, M. Ali, H. Batra, N. Bansal, D. Saluja; Medical Biotechnology Lab, DR B R Ambedkar Cente for Biomedical Sciences, Delhi University, Delhi, India

MTG family of proteins is a family of corepressors consisting of MTG8 (Myeloid translocation gene 8), MTGR1a Myeloid translocated gene related 1a) and MTG16 (Myeloid translocation gene 16) that are highly conserved during evolution. All members have several spliced forms and are found in fetal and adult tissues. These proteins have been implicated in gene regulation during fetal brain development. Though MTG8 and MTG16 have been shown to be translocated in t(8:21) and t(16:21) respectively leading to different types of myeloid leukemia, translocation involving MTGR1a has not been observed till date. To understand the function of MTGR1a in particular and MTGs in general two approaches were used. The function of MTGR1a was checked in relation to the proteins it interacts with and also by analysis of the gene expression profile in human embryonic kidney (HEK) cells over expressing MTGR1a protein. Using the yeast two hybrid technique it was suggested that MTGR1a interacts with the human Sin3B (hSin3B)protein which is a global transcription regulator. Deletion mutants of

MTGR1a when cotransformed with hSin3B indicated that the N terminal of MTGR1a interacts with hSin3B. MTGR1a protein like hSin3B does not have a DNA binding domain and therefore it is envisaged that regulation of genes by MTGR1a takes place by binding of MTGR1a to a transcription factor on one hand and recruitment of the hSin3b transcription regulator takes place thereafter. To elucidate the genes regulated by MTGR1a, it was over expressed in HEK cell line and a microarray experiment was performed.Of the many biochemical pathways effected, the MAP kinase pathway was analysed in detail. Data is presented to show that the expression of key genes of the MAP kinase pathway are effected as a result of MTGR1a over expression.

1273/B483

Purification of MBP- and Strep II-tagged Proteins.

A. Heijbel, M. Carlsson, A. Karlsson; Ge Healthcare, Uppsala, Sweden

Recombinant tagged proteins can be purified to high purity in one step using affinity chromatography. The tag can either be a peptide or a protein, and the choice of tag depends on target protein characteristics and the aim for purifying the protein. Two new chromatography media are presented, StrepTactin™ Sepharose™ High Performance that utilizes that the small 8 amino acid peptide, *Strep* II-tag, has high affinity for the Strep-tactin ligand. Dextrin Sepharose High Performance, on the other hand is based on the protein tag, Maltose binding protein (MBP, Mr: ~42 000), which binds specific to carbohydrates (e.g. dextrin). The specificity of the two media towards their respective tag gives high purity of the target proteins. In this study, simple protein purifications on ÄKTAdesign™ chromatography systems using MBPTrap™ HP (prepacked with Dextrin Sepharose High Performance) and StrepTrap™ HP,(prepacked with StrepTactin Sepharose High Performance) are presented. Purifications were also scaled-up giving equivalent results. Robustness was tested by running six repeated purifications of a *Strep* II-tagged protein expressed in E. coli on the same StrepTrap HP column including regeneration with 0.5 M NaOH between each run. No tendencies in reduction of final purity and yield of the target protein were observed, demonstrating the high reproducibility during repeated usage of StrepTrap HP. In summary, the results show the ease of using prepacked StrepTrap HP and MBPTrap HP columns together with chromatography systems. Equivalent results are obtained when scaling up showing the simplicity to purify larger quantities of pure target protein. Results also show that StrepTrap HP can be efficiently regenerated repeated times with 0.5 M NaOH without losing binding capacity.

1274/B484

Nanoscaled Ca+2 Functionalized CdS Quantum Dots for Early Heart Attack Prevention.

F. J. Zayas; Chemistry, University of Puerto Rico, Mayagüez Campus, Mayagüez, PR

These experiments were designed with the purpose of functionalizing CdS quantum dots with Ca+2. The motivation for engineering CdS quantum dots functionalized with calcium is for detecting the presence of troponin within an environment. Troponin is a calcium binding protein. Its detection is crucial in early detection of heart attacks. The CdS quantum dots synthetisized, absorb light at 280 nm and between 300 and 400 nm. The Ca+2 has little effect on the 300 nm absorption by the quantum dots. However, the absorption of light above 400 nm increases with the amount of calcium added to CdS containing solution. When the absorbance of the CdS quantum dots increased as the calcium concentrations increased, there came to that there was an interaction between CdS and (Ca+2). This interaction was demonstrated in the data of absorbance gathered.

1275/B485

Detection of Post Translational Modifications Using In Situ Proximity Ligation Assay (In Situ PLA).

E. Nystrom, A. Andersson, A. Reyes, G. Holmquist, G. Edfeldt, M. Gullberg; Olink Bioscience, Uppsala, Sweden

In situ Proximity Ligation Assay (*in situ* PLA) enables the study of protein modifications and interactions in fixed cells and tissue. We present a generic approach to *in situ* PLA using secondary antibodies. The secondary antibodies are conjugated with DNA strands and the conjugates are called PLA probes. When bound in proximity, due to source specific binding to their respective primary antibody, the DNA strands of two PLA probes can act as templates for two connector oligonucleotides. After ligation, the two connector oligonucloeotides form a circular DNA molecule. This circular DNA is then amplified in a process called rolling-circle amplification (RCA). A long concatemeric single stranded DNA molecule is formed which represents an individual protein molecule. By hybridizing multiple fluorescently labeled oligonucleotide probes to the rolling-circle product, it can be visualized as a distinct dot in a standard fluorescence- or confocal microscope. We present data on the relative quantification of phoshporylated Epidermal Growth Factor Receptor (EFGR) on fixed cells and Formalin Fixed Paraffin Embedded (FFPE) tissue using *in situ* PLA. After stimulation of A431 cells with EGF we can monitor the phosphorylation status. In addition, we detect different levels of phsosphorylated EGFR on FFPE tissue such as skin and placenta. By combining the requirement of dual antibody recognition of the target (e.g. post translational modification or protein complex) with signal amplification, *in situ* PLA exhibits both high sensitivity and specificity.

1276/B486

Development of Quantitative Multiplex Immuno-QPCR.

K. Felts, B. Hsue, C. Monell; Agilent Technologies, La Jolla, CA

A technique for antigen detection, called immuno-PCR, was developed by Sano et al. in 1992. It combines the molecular recognition of antibodies with the high DNA amplification capability of PCR. The procedure is similar to conventional enzyme-linked immunosorbent assays (ELISA) but allows for more sensitive detection and lower sample volumes. Instead of an enzyme, a DNA molecule is linked to the detection antibody and serves as a template for PCR. Real-time PCR (QPCR) provides accurate quantitation over a large dynamic range and has exquisite sensitivity. When applied to immuno-PCR QPCR makes it possible to quantify very low amounts of DNA-coupled detection antibody with high accuracy. Success using this method is dependent on consistent production of antibody-DNA conjugates, background reduction measures, and optimization of the DNA amplification conditions. Reagents and methods were developed and were shown to enable simultaneous detection of multiple protein analytes using biotinylated antibodies, mouse antibodies, and rabbit antibodies in immuno-QPCR assays.

1277/B487

Enzyme-linked Immuno PCR.

A. Belyaev, K. Felts, C. Monell; Agilent Technologies, San Diego, CA

Immuno PCR (IPCR) can provide for significant increases in sensitivity compared to standard ELISAs using the same antibodies. However, traditional IPCR applications face several hurdles including cumbersome reagent manufacturing, limited compatibility with existing ELISA formats, and a complex intellectual property landscape. We describe enzyme-linked immuno PCR (EL-IPCR), a pioneering immuno PCR approach that combines the high sensitivity of immuno PCR, convenient reagent production processes, and applicability to existing ELISA kits. Similar to ELISA, an enzyme (a polymerase for example) is linked to a detection antibody. However, instead of adding chromagenic, chemiluminescent, or fluorogenic detection substrates as in traditional ELISA, a modified oligonucleotide substrate is added. The substrate contains 2' O-Methyl modified RNA (2'OMe) and deoxyuridine (dU) modified nucleotides. The linked polymerase, such as Klenow fragment of DNA polymerase I, creates a copy of the substrate using normal A, C, T, G nucleotides resulting in a DNA strand that lacks modifications. The copy, but not the modified oligonucleotide, is amplified in QPCR using a Pfu polymerase that can easily amplify the copy, but not the original modified substrate. We demonstrate that the modified oligonucleotide template can be copied in solution by an anchored Klenow probe. We also show that the Klenow probe and a modified oligonucleotide probe can be effective when acting in proximity, both in solid phase and homogenous immuno-QPCR assays.

1278/B488

Development of a Novel Method to Isolate Genomic DNA, RNA and Protein from Single Undivided Biological Sample.

R. Dhulipala, R. Bruno, C. Cai, K. M. Kapolka, M. Jiang; R & D, GE Healthcare, Piscataway, NJ

Functional genomics and biomarker studies require high quality of nucleic acid and protein samples to serve as probes and targets. Until recently researchers employed separate kits to isolate DNA, RNA and Proteins. Recent studies related to molecular genetics warrant good correlation between gene/transcript expressions, protein expression, copy number variation and SNP detection. Use of different samples to isolate different bio molecules may adversely skew the results due to presence of heterogeneity between cell and tissue samples. GE Healthcare, a leader in protein purification and sample preparation, recently developed a novel method to simultaneously isolate high quality genomic DNA, RNA and protein from a single undivided source of tissue or cells sample in less than one hour. The performance of the method was evaluated by using animal tissues from different organs (such as liver, kidney, spleen, brain, lung and intestine) or various cultured mammalian cell lines (HeLa, NIH-3T3, CHO-K1 and HEK-293). In this presentation we showed that the bio molecules isolated by this novel method can be used successfully in various applications such as PCR, Restriction digestion, sequencing and array CGH (gDNA); RT-qPCR and gene expression analysis (RNA) and SDS-PAGE, Western blotting, 2D DIGE and LCMS (Protein).

1279/B489

Purified CyaC-acyltransferase Cloned from Bordetella Pertussis Catalyzes the Hydrolysis of Synthetic Substrates In Vitro.

N. Thamwiriyasati[1], B. Powthongchin[1,2], C. Angsuthanasombat[1]; [1]Molecular Genetics and Genetic Engineering, Institute of Molecular Biology and Genetics, Salaya Campus, Mahidol University, Budhamonthol, Thailand, [2]Biopharmacy, Silpakorn University, Budhamonthol, Thailand

Bordetella pertussis adenylate cyclase-haemolysin toxin (CyaA) is a unique RTX (Repeat-in-ToXin) pore-forming toxin that requires post-translational fatty-acyl modification. Recombinant CyaA pore-forming (CyaA-PF) fragment expressed in Escherichia coli was previously shown to be in vivo palmitoylated by the co-synthesized CyaC-acyltransferase to exert haemolytic activity. In this study, the 21-kDa CyaC was separately expressed in E. coli as inclusion bodies that were solubilized in 8 M urea and successfully refolded into an enzymatically active monomer. In addition to efficiently activating CyaA-PF in vitro, the refolded CyaC-acyltransferase was able to hydrolyze p-nitrophenyl acetate and p-nitrophenyl palmitate similar to chymotrypsin. This suggests that CyaC may share a similar mechanism of hydrolysis with chymotrypsin-like proteases.

1280/B490

New Codon-Optimized and Stabilized Luciferase from Luciola Cruciata Exhibiting Red Luminescence.

D. J. Coleman, G. M. Cook, J. J. Naleway; Research and Development, Marker Gene Technologies, Inc., Eugene, OR

Bioluminescence is the light produced in certain organisms as a result of enzyme mediated oxidation of a luciferin substrate often mediated by adenosine triphosphate (ATP). A codon optimized and stabilized luciferase gene based upon the sequence of the natural luciferase gene isolated from *Luciola cruciata* (Japanese firefly) has been developed. Optimization for expression in human cell lines was accomplished by removing certain cryptic splice acceptor sites, palindromic sequences and modifying insect codons for optimization of expression in human cells. In addition, an amino acid modification of Ser encoded at nucleotide position 875-877 to Tyr was performed. This new gene codes for a modified amino acid sequence which exhibited improved expression levels in mammalian cells, as well as shifted emission wavelengths (green to red) and improved thermal stability. Expression vectors, pDC57 and pDC99 containing the cytomegaloviral (CMV) and simian viral (SV40) promoter elements respectively were prepared. Both of these new luciferase vectors were transfected into mammalian cells and exhibited long-wavelength light emission using the common, inexpensive substrate D-luciferin, as well as improved thermostability (>100X after 1 h at 25°C and >1000X after 2h at 37°C) and higher expression levels in mouse endothelial fibroblast cell line NIH3T3 compared with the wild-type luciferase sequence from pSV40-GL3. The Luciola cruciata luciferase expression was used as a genetic marker in cell extracts when mixed with substrates (D-luciferin, Mg^{2+}, ATP, and O_2), and the resulting luminescence measured using luminescent detection. Isolation of the luciferase protein from both mammalian and bacterial cells was performed by culturing into transgenic *E. coli* JM109 or lipofection into NIH3T3 cells and collecting and purifying the luciferase from the culture medium.

1281/B491

Testing Single-Nucleotide Polymorphisms with Humanized Yeast.

N. Ko[1], J. P. Tam[1], T. Stearns[1, 2]; [1]Biology, Stanford University, Stanford, CA, [2]Genetics, Stanford Medical School, Stanford, CA

Genetic variations in the human genome play a key role in phenotypic variation, including genetic predisposition to diseases and responses to therapeutic drugs. Single-nucleotide polymorphisms (SNPs) are a type of genetic variation, in which a single nucleotide is changed relative to a reference sequence. SNPs that result in an amino-acid change in a protein are non-synonomous coding SNPs. To date, many SNPs have been identified in the human genome, but most have not been tested for alteration of function of the genes in which they occur. This problem is complicated by the difficulty of assessing SNP allele function directly in human cell lines. We aim to comprehensively test non-synonymous coding SNPs using "humanized" yeast. In our approach, we complement deletion alleles of yeast genes with the orthologous human gene. Known SNP variants in the human gene are then introduced to determine their effect on function. Here we report findings with three pilot human gene candidates: PIGB, PIGZ, and ERCC2. The PIGB and PIGZ proteins are involved in the synthesis of glycolipids that anchor proteins to the cell surface, and ERCC2 is a helicase that repairs DNA damage via the nucleotide excision repair pathway. These genes have been chosen because they contain non-synonymous coding SNPs that have been validated by the International HapMap Project, and previous studies have shown that they can complement deletions of the yeast orthologs. We have confirmed the ability of the human reference sequence alleles to complement yeast deletions and are now assessing the function of the validated SNP alleles by accurately determining growth properties of the humanized yeast strains with and without stresses relevant to the function of these particular gene products.

1282/B492

NADPH Oxidase Mediates IL-1β and TGF-β1 Expression by Regulating JAK/STAT3 and NF-κB Signaling in Cerulein-stimulated Pancreatic Acinar Cells.

K. Ju[1], J. Lim[2], K. Kim[1], H. Kim[3]; [1]Department of Pharmacology,Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, South Korea, [2]Institute of Gastroenterology, Yonsei University College of Medicine, Seoul, South Korea, [3]Department of Food and Nutrition, Brain Korea 21 Project, Yonsei University College of Human Ecology, Seoul, South Korea

Reactive oxygene species (ROS) are involved in NF-kappaB activation, cytokine expression and thus, pathogenesis of pancreatitis. Recently, we demonstrated that activation of NADPH oxidase is induced by cerulein, a cholecystokinin analogue in pancreatic acinar AR42J cells, which mediates the production of ROS. In present study, we investigated the role of NADPH-oxidase in cerulein-induced signaling cascade leading to gene expression in pancreatic acinar AR42J cells. We found that Janus kinase 2 (Jak2)/signal transducer and activator of transcription (Stat) 3 pathway was activated by cerulein. Cerulein-induced activation of Jak2/Stat3 and NF-κB were inhibited by the treatment of NADPH oxidase inhibitor DPI and transfection with AS ODNs for NADPH oxdase subunits in AR42J cells. Cerulein-induced expressions of IL-1β and TGF-β1 were also suppressed by DPI treatment and transfection with AS ODNs for NADPH oxidase subunits. Additionally, ceruelin-induced IL-1β and TGF-β1 expressions, and NF-κB activation were inhibited by Jak2 inhibitor AG490, indicating that the expression of IL-1β and TGF-β1 as well as NF-κB activation are mediated by activated Jak2. The results suggest that cerulein-induced expression of inflammatory cytokines IL-1β and TGF-β1 is mediated by Jak2/Stat3 and NF-κB, which is related to NADPH oxidase in pancreatic acinar cells.

1283/B493

Self-Fermented Pine Needle Extracts (SFPEs) Improves the Effects of Blood Circulation.

J. Jung[1], D. Lim[2], H. Cheong[1]; [1]Biotechnology, Chosun University, Gwangju, South Korea, [2]Medicine, Chosun University, Gwangju, South Korea

Biological activity of plant extract is the essence for traditional medicine, which uses the pharmacological efficacy of natural compounds present in pine needle for treating human diseases. A form of extract, isolated from needles of Pinus densiflora Sieb. et Zucc., was tested for various effects on fibrinolytic activity, blood plasma cholesterol, and on microbes. The extract has mixtures of compounds having diverse effects, their composition change during self-fermentation. Among crude, Ethanol cut-off and buthanol cut-off SFPEs, ethanol and butanol cut-off SFPEs found effective fibrinolytic acrivity than the crude extract. The experiment showed that fresh pine needle extract (PE) , SFPEs , 1,8-cinoele and Camphor can retard growth of bacteria Salmonella typhimurium, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa and Bacillus subtilis. And if also showed that the strains are highly sensitive to SFPE7, 1,8-cineole and camphor. The SFPE7 (0.15 mg/ml and 0.3 mg/ml) caused relaxation of spontaneous contraction and relaxation rhythm of thoracic arterial tissues from rat. We found out that SFPEs decreased cholesterol and triglyceride levels in cholesterol-fed rats. Therefore, SFPE might be useful in improving blood circulation and could be a good source of functional food development.

1284/B494

Simultaneous Protein Expression by a Lentiviral Bicistronic Vector Based in the 1D/2A Sequence of Foot-and-Mouth Disease Virus (FMDV).

V. I. Torres[1], F. A. Garces[1], K. R. Ordenes[1], S. A. Leal[2], C. C. Garner[2], P. L. Zamorano[1]; [1]Facultad de la Salud, Universidad de Antofagasta, Antofagasta, Chile, [2]Psychiatry and Behavioral Sciences, Stanford University, Palo Alto, CA

OBJECTIVE. Lentiviral vectors are of great utility in neurobiology due to its capacity of integrate into genomic DNA and express proteins in post-mitotic cells. However, its cargo capacity is limited to 9.5 kb of packed DNA. This disadvantage is marked in bicistronic lentiviral vectors based in IRES sequences, since the size of classical IRES sequences is about 600 bp. Here we displayed a bicistronic lentiviral vector, based in 1D/2A sequence of the foot and mouth disease virus (FMDV) that allows the expression of EGFP as a reporter and the protein of interest labeled with a HA tag. METHODS. The FUGW plasmid was modified by introducing the construct EGFP-1D/2A-HA- PCS downstream of the ubiquitin promoter. The effectiveness of the 1D/2A sequence to co-express two proteins was evaluated in HEK293-T cells by immunofluorescence and Western blot directed to EGFP and the subunit of the exocyst complex, Exo70. RESULTS. The sequence 1D/2A allowed the co-expression of EGFP and Exo70 with high efficiency in HEK293-T cells with a low percentage of fused EGFP-Exo70 proteins. CONCLUSIONS. This vector allows to monitoring the infection efficiency of the virus with EGFP expression and the protein of interest could be detected by immunoflurescence towards HA tag. The 96 bp of the 1D/2A sequence shorter in size that the traditional IRES

sequence, it would permit the expression of larger proteins in lentiviral vectors. Supported by DirInv Projects #1315, #1314, University of Antofagasta. Fondecyt #1070462, Conicyt, Chile.

1285/B495

Global Gene Expression Profiling of Single Cells using Oligonucleotide Arrays.

C. Wong[1], R. Desai[2], R. Raja[1,2], R. Reijo Pera[1]; [1]Stanford University, Palo Alto, CA, [2]MDS Analytical Technologies, Sunnyvale, CA

Microgenomics refers to the field of genomics that involves precise molecular analysis of single or small groups of cells, typically isolated by methods such as laser capture microdissection. Current whole genome expression profiling methods require the pooling of isolated cells to obtain enough RNA for downstream analysis. However, studies show that many homogenous samples actually consist of cells with different gene expression profiles. Moreover, certain rare samples, such as patient biopsies, are only available in extremely small quantities. Thus, the ability to perform single cell gene expression analysis at the whole-genome level will enable exploratory studies using such samples. The small amount of total RNA found in a single cell, typically between 10 - 40 picograms (pg) in most mammalian cell types, poses a challenge for such analysis. To address this critical research need, MDS Analytical Technologies recently developed and optimized a novel process that integrates the efficient isolation of total cellular RNA from a single cell, linear amplification of isolated RNA, and labeling of amplified RNA (aRNA) for microarray analysis or quantitative real-time PCR. We tested the amplification potential of the new RiboAmp ULTRA RNA Amplification kit on RNA isolated from single human cells, crude lysates from single human cells and purified human reference RNA diluted to single cell level. We found that amplification of RNA from single cells typically yielded between 15 to 30 µg of aRNA, equivalent to over 10 million fold amplification. Hybridization of the amplified RNA from single human embryonic stem cells onto Affymetrix HU133 Plus 2.0 human arrays resulted in 26-32.0% of percent present calls, whereas the hybridization of the amplified negative control only yielded 1.9% of percent present calls. Microarray analysis of independently amplified single cells of the same lineage also showed extremely high correlation between samples, indicating the reproducibility and fidelity of the amplification process. Together, these data suggest that the RiboAmp ULTRA RNA Amplification kit is a robust and reliable method for the study of single cell or small sample gene expression analysis.

1286/B496

Gene Expression Profiling from a Single Cell.

R. Desai[1], C. Vanderburg[2], S. Blakely[1], S. Chu[1], A. Munishkin[1], V. Dave[1], S. C. Miller[1]; [1]MDS Analytical Technologies, Sunnyvale, CA, [2]Massachusetts General Hospital, Charlestown, MA

Microgenomics refers to the quantitative genomic molecular analysis of single cells or small groups of cells, typically isolated using precise collection methods such as laser capture microdissection (LCM). Current whole genome expression profiling methods require that researchers pool the isolated cells in order to obtain enough RNA for downstream analysis. Several studies suggest that pooled cell samples, thought to be homogenous, are often composed of cells with quite different gene expression profiles. It is therefore desirable to perform experiments on a single cell. However, the small amount of total RNA found in a single cell poses a challenge to perform whole genome expression profiling. To address this critical research need, MDS Analytical Technologies recently developed and optimized a novel process that integrates the efficient isolation of total cellular RNA from a single cell, linear amplification of isolated RNA, and labeling of amplified RNA (aRNA) for microarray analysis and quantitative real-time PCR (qRT-PCR). Here we demonstrate that a single cell can be used with a high level of confidence to study gene expression levels using common analysis platforms. As little as 10 picograms of RNA, the approximate amount in a single microdissected cell, were amplified using the new RiboAmp ULTRA RNA Amplification kit from MDS Analytical Technologies. The RNA was amplified up to 10 million fold in two rounds of amplification, labeled and then hybridized to multiple microarray platforms. High concordance was found between replicate hybridizations and between amplified and unamplified RNA, demonstrating high reproducibility and fidelity of the amplification process.

1287/B497

Rapid and Sensitive PCR-Based Method for Site-Specific DNA Mutation Detection.

M. El-Mogy[2], Y. Haj-Ahmad[2,1]; [1]Norgen Biotek Corp., Thorold, ON, Canada, [2]Brock University, St. Catharines, ON, Canada

Mutation detection is important for examining DNA quality and suitability for different applications including mutagenesis studies. The conventional methods for mutation detection are based on monitoring the biological activity of the expressed proteins, which is not applicable to regulatory sequences, or by DNA sequencing. Both of these methods are time consuming and impractical for high throughput screening of mutation rates and frequencies. In this study we report on the development and evaluation of a rapid and simple PCR-based procedure for the detection of mutations. The method worked very efficiently with both eukaryotic and prokaryotic cells. Using this method we were able to detect the proportional increment in mutation rate with increasing EtBr concentrations. Furthermore, we were able to precisely detect the number of mutated DNA molecules in a given volume of DNA. This PCR-based method is rapid, highly sensitive and quantitative.

1288/B498

High Level of Expression, Purification and Characterization of Active rKM+ Lectin in E. coli Hosts.

M. Pranchevicius[1,2], L. Oliveira[3], N. Avanci[1], J. Rosa[3,4], A. Quiapim[1], V. Cossalter[1], M. Brito[1], E. Aragão[5], T. Ferreira[5], M. Barreira[3], M. Goldman[1]; [1]Department of Biology, FFCL/USP, University of São Paulo, Ribeirão Preto, Brazil, [2]School of Medicine, UFT, Federal University of Tocantins, Palmas, Brazil, [3]Department of Molecular and Cell Biology, FMRP/USP, University of São Paulo, Ribeirão Preto, Brazil, [4]Protein Chemistry Center, FMRP/USP, University of São Paulo, Ribeirão Preto, Brazil, [5]Department of Chemistry, FFCLRP/USP, University of São Paulo, Ribeirão Preto, Brazil

KM+ is a D-mannose-binding lectin from *Artocarpus integrifolia* seeds that induces interleukin (IL)-12 production by macrophages and protective T helper 1 immune response against *Leishmania major*, *Leishmania amazonensis*, and *Paracoccidioides brasiliensis* infections. Considering its potential use as an immunotherapeutic molecule, this study was designed to produce high-level of active rKM+, in *E. coli* system, for both research and pharmaceutical purposes. The KM+ cDNA was inserted in pET29a+ vector and expressed in *E. coli* strains

BL21(DE3), BL21(DE3) Codon Plus-RP and BL21(DE3) Rosetta. The cells were induced under the given optimized conditions, 0.5mM IPTG at 20°C for 19h, and resulted in the accumulation of large amounts of soluble rKM+ in the three *E. coli* strains. The rKM+ proteins were functionally active, as shown by one-step purification through immobilized D-mannose affinity chromatography, which resulted in 22.4 mg/l, 17.9 mg/l and 14.1mg/l of purified rKM+ for BL21 (DE3) Codon Plus-RP, BL21(DE3), BL21(DE3) Rosetta, respectively. The apparent molecular mass of the native KM+ and rKM+s were estimated by gel filtration and showed that the native protein is a tetramer and the rKM+s are monomers. The exact molecular weight and the identity were verified with mass spectrometry analysis and N-terminal sequencing by automated Edman degradation. To verify whether the soluble rKM+s have folded in a similar way to the native KM+, we characterized the rKM+s by circular dichroism (CD) and fluorescence spectroscopy. The CD spectrum of rKM+s indicated that the global fold comprised β-sheet structure and the fluorescence analysis are in accordance with our CD results indicating that rKM+s are correctly folded, stable and have a defined 3D structure suitable for functional studies. Our data provides evidences that *E. coli* expression system can be used to produce large quantities of functional KM+ for research and industrial purposes. Financial support from FAPESP.

1289/B499

Signaling in Sepsis: Using Peptide Reporters for Early Systems Analysis of Kinase Activation in the ATRA-Differentiated HL60 Response to LPS.

J. E. Sylvester[1], S. Kron[2,3]; [1]Biochemistry and Molecular Biology, The University of Chicago, Chicago, IL, [2]Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL, [3]Ludwig Center for Metastisis Research, The University of Chicago, Chicago, IL

Despite the gravity and national expense of septic shock, its underlying mechanisms and potential effective treatments remain unknown. Attempts to interrupt the development of sepsis through the inhibition of a single mediator have been unsuccessful. The pathogenic mechanisms are complex and a multivariate treatment based on the heterogeneity of cellular responses to stress is expected to be more effective. The goal of our research is to define the early dynamics of intracellular molecular signatures in the septic response using a systems analysis of protein expression and modification in lipopolysaccharide-stimulated, retinoic acid-differentiated HL60 cells. Using patterns of kinase activities to follow characteristic signaling pathways, we have implemented two improved methods to simplify conventional phosphoproteomic analyses: 1) Measuring the activity of kinases through quantitative measurements of immobilized synthetic substrate phosphorylation using BioRad's Luminex system 2) Comparative analysis against endogenously phosphorylated substrates isolated from cell lysates using Invitrogen's ProQ Diamond® resin, followed by phosphoproteomic analysis using LTQ-Orbitrap Hybrid FT MS/MS. Towards developing a clinically useful assay, we have compiled a set of synthetic peptide substrates for early response kinases in the Toll-like receptor 4 signaling cascade, including AKT, PI3K, and c-SRC, and monitored their phosphorylation in vitro. Endogenous signaling targets have been highlighted through phosphoproteomic analysis and a hierarchical organization of the Toll-like receptor 4 signaling cascade is proposed.

1290/B500

Dual Detect CELISA Assay-A Convenient Cell-Based ELISA to Measure Both Total and Phosphorylated Proteins an a Single Well.

H. Liu, A. Butler, J. Archdeacon; R&D, Millipore, Temecula, CA

OBJECTIVE & BRIEF METHOD Dual Detect CELISA is a simple and efficient cell-based ELISA assay to measure both total and phosphorylated targets in whole cells in a 96 well microplate format using dual fluorescent detection. Cells are cultured directly in the microplate and stimulated as desired. They are then fixed and simultaneously incubated with two primary antibodies. One is a phospho-specific antibody directed to the phosphorylated epitope that correlates with its activation and the other is a total antibody that recognizes the target-protein, regardless of phosphorylation status. Both the total target protein and the phosphorylated protein is measured in a single well using a double immunoenzymatic labeling procedure, either horseradish-peroxidase (HRP) or alkaline phosphatase (AP), and two spectrally distinct fluorogenic substrates for HRP and AP. The fluorescence of the phosphorylated protein is normalized to the total protein in each well thus reducing well-to-well variations. RESULT Six targets are currently offered from Millipore using Dual Detect CELISA assay. After total antibody normalization, phospho-Akt (Ser473) showed over 2-fold excess in PDGF (50ng/mL, 5minutes) stimulated NIH3T3 cells than non-stimulated cells. Phospho-Erk1/2(Thr202/Tyr204)(Thr185/Tyr187) got over 10-fold in NIH3T3/PDGF cells. In A431/EGF (100ng/mL for 5 minutes) cells, phospho-EGFR (Tyr1173) showed over 2-fold, phospho-ErbB2 (Tyr1248) got over 3-fold. In HeLa/Anisomycin (25µg/mL, 30 minutes) cells, phospho-p38 (Thr180/Tyr182) showed over 2-fold. In NIH3T3/Anisomycin (25µg/mL, 30 minutes) cells, phospho-ATF2 (Thr69/71) got over 2-fold. SUMMARY Millipore's new Dual Detect CELISA is a simple and efficient cell-based ELISA assay to measure both total and phosphorylated targets in whole cells in a 96 well microplate format using dual fluorescent detection. This assay eliminates the need to prepare cell lysates and can be used to investigate phosphorylation levels among cell and/or growth condition differentials. The fluorescence of the phosphorylated protein is normalized to that of the total protein in each well, allowing for the correction of well-to-well variations.

Structure of the Nuclear Envelope I (1291 – 1309)

1291/B504

Nuclear Shape and Integrity in the Closed Mitosis of Fission Yeast Depend on the Ran GTPase System, the Spindle Pole Body and the Endoplasmic Reticulum.

Y. Gonzalez[1], K. Meerbrey[1, 2], J. Chong[1], Y. Torii[1, 2], N. Padte[4], S. Sazer[1, 2, 3]; [1]Department of Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX, [2]Graduate Program in Cell and Molecular Biology, Baylor College of Medicine, Houston, TX, [3]Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, [4]Microbiology Department, Columbia University College of Physicians and Surgeons, New York, NY

In the open mitosis of animal cells the nuclear envelope breaks down, but during closed mitosis in yeast it remains intact. In fission yeast the two spindle pole bodies are embedded in the nuclear envelope where they nucleate assembly of an intranuclear mitotic spindle, which drives nuclear shape changes as it elongates. During this symmetrical nuclear division the nuclear volume remains constant but the nuclear area

increases by 26%. This study is aimed at understanding how the yeast nucleus grows, changes shape, and divides symmetrically while resisting the pushing forces of spindle elongation and how the Ran GTPase system influences mitotic specific nuclear envelope changes during closed mitosis. When the Ran GTPase system is compromised, nuclear division in fission yeast is asymmetrical and the nuclear envelope ruptures during mitotic spindle elongation. In this asymmetric division the newly synthesized spindle pole body is preferentially associated with the smaller nucleus, suggesting that SPB structure and/or function depend on the Ran GTPase and that the SPB influences nuclear size and shape changes during closed mitosis. The nuclear envelope breaking is partially rescued by slowing spindle elongation with the atb2-983 mutation in α2-tubulin, by stimulating endoplasmic reticulum membrane proliferation by overexpression of the endoplasmic reticulum membrane protein HMG Co-A reductase, or by changing endoplasmic reticulum membrane conformation by deletion of the reticulon-encoding gene rtn1. These observations suggest that the endoplasmic reticulum is essential for nuclear envelope growth, most likely by providing a direct lipid reservoir. Taken together these results demonstrate that the Ran GTPase system is required to coordinate spindle elongation with lipid addition to the nuclear envelope during closed mitosis. The Ran GTPase system may be involved in the redistribution of lipid within the continuous nuclear envelope/endoplasmic reticulum membrane system during both open and closed mitosis.

1292/B505

Probing Nucleocytoplasmic Transport with Fluorescence Fluctuation Spectroscopy and Two-photon Activation of Photoactivable GFP.

Y. Chen[1], B. Wu[1,2], J. Mueller[1]; [1]Physics, University of Minnesota, Minneapolis, MN, [2]Albert Einstein College of Medicine, New York, NY

Large proteins and macromolecular complexes have to enter and leave the nucleus in an efficient and selective manner. Macromolecules that are greater than 40 kD are transported actively across the nuclear envelope through nuclear pore complexes using soluble transport factors or carrier molecules that cycle between the cytoplasm and nucleus. The carrier proteins themselves interact with each other in order to transport cargo proteins across the nuclear pore complexes. In this work, we apply dual-color time-integrated fluorescence cumulant analysis (TIFCA), a fluorescence fluctuation spectroscopy technique, to investigate the protein interactions of the carrier proteins directly in cells. In addition, we apply two-photon activation to directly examine the nucleocytoplasmic transport of photoactivable GFP tagged carrier proteins. With these two approaches, we are able to probe the nucleocytoplasmic transport process of NTF2 directly inside cells and under equilibrium conditions. We investigate the oligomerization of NTF2 in cells and its transport properties when crossing the nuclear pore complexes. The experiments identify the presence of NTF2 dimers in the cytoplasm and nucleus of cells, while the measured transport properties across the nuclear pore complex are not consistent with dimeric NTF2. We will discuss the implications of these results for functions of nuclear envelope and models of nucleocytoplasmic transport.

1293/B506

The Nuclear Pore Complex of the Ciliated Protozoan Tetrahymena thermophila: Its Distinct Composition between Two Functionally-Distinct Nuclei and Possible Contribution to Nuclear Differentiation.

M. Iwamoto[1], F. Bunai[1], Y. Hiraoka[1,2,3], T. Haraguchi[1,2,3]; [1]Kobe Advanced ICT Research Center, National Institute of Information and communications Technology, Kobe, Japan, [2]Graduate School of Frontier Biosciences, Osaka University, Suita, Japan, [3]Graduate School of Science, Osaka University, Toyonaka, Japan

The ciliated protozoa have two functionally and structurally distinct nuclei in a single cell. A somatic macronucleus (MAC) is transcriptionally active while a germ line micronucleus (MIC) is inert during vegetative growth. Although understanding such "nuclear dimorphism" is a central issue unique to ciliate biology, molecular bases of nuclear differentiation have remained largely unknown. To understand molecular bases of nuclear differentiation in ciliates, we study structural difference of the nuclear pore complex (NPC) between two nuclei in *T. thermophila*. We identified 13 nucleoporins, including Seh1, Nup93, Nup96, gp210, Nup155 and four Nup98s, based on localization of GFP fusions. While most Nups were localized to both of MAC and MIC, interestingly four Nup98s showed strikingly differentiated localization. Two out of four Nup98s possessing GLFG repeats were localized exclusively to MAC and the other two possessing novel NIFN repeats instead of GLFG exclusively to MIC in vegetative growing cells. However, at nuclear differentiation during sexual reproduction one micronuclear Nup98 is unexpectedly temporary localized to developing new MAC, but not to new MIC. These facts suggest that particular nucleoporins or the NPC might be involved in nuclear differentiation. To further understand involvement of the NPC in nuclear differentiation, we examined dynamic behavior of the NPC during sexual reproduction using the method of fluorescence live-cell imaging, in combination with electron microscopy, to monitor dynamic behavior of GFP-tagged nucleoporin in living *Tetrahymena* cells. We found that the NPCs moved toward the anterior side of the nuclear envelope during second postzygotic mitosis to generate distinguishable nuclei in a number of the NPC. Live-cell imaging further showed that these NPC-rich and NPC-poor nuclei were destined to become a new MAC and MIC, respectively. These results suggest that the NPCs, or some components of the NPC, may contribute to nuclear differentiation through regulation of selective nuclear transport.

1294/B507

Nuclear Pore Complexes Form at a Constant Rate Independently Of DNA Replication in Interphase of Live Mammalian Cells.

E. Dultz, J. Ellenberg; Gene Expression Unit, EMBL, Heidelberg, Germany

Nuclear pore complexes mediate all traffic between the nucleus and the cytoplasm. They are large protein complexes of 125 MDa in vertebrates composed of about 30 different proteins which are called nucleoporins. In cycling mammalian cells assembly of new nuclear pore complexes occurs in two phases of the cell cycle: after mitosis when the entire nuclear envelope is reassembled around the daughter chromatin masses and in interphase when the nucleus grows. While we have recently analyzed the kinetics of postmitotic nuclear pore complex assembly in living cells, very little is known about the assembly process of nuclear pore complexes into an intact nucleus in interphase. We have therefore now analyzed the dynamics of interphase nuclear pore complex assembly in living mammalian cells. We have developed assays to follow living cells throughout complete cell cycles and imaged their nuclei at single pore resolution. The density of pores in the nuclear envelope was determined to be constant throughout G1, S and G2 phase at 6-7 NPCs per μm^2. The nuclear surface area grew linearly and approximately doubled its size in one interphase. Blocking DNA replication with aphidicolin did not affect NPC density or the

rate of nuclear growth but by contrast allowed nuclei to grow to very large sizes because reentry into mitosis was prevented. In summary, our data indicates that nuclear pore complexes are assembled at a constant rate during interphase and that NPC assembly and nuclear growth are independent of DNA replication.

1295/B508

Electrostatic Interactions Appear to Contribute to Nuclear Pore Selectivity.

L. Colwell[2], M. Brenner[2], K. Ribbeck[1]; [1]Center for Systems Biology, Harvard University, Cambridge, MA, [2]School of Engineering and Applied Sciences, Harvard University, Cambridge, MA

Nuclear pore complexes (NPCs) act as selective filters, which control the exchange of molecules between the nucleus and the cytoplasm. They allow efficient translocation of nuclear transport receptors and transport receptor-cargo complexes, while restricting the passage of many other molecules. The exact mechanism of selective translocation through the NPC is not yet fully understood. To address this question we used a bioinformatics approach to compare the basic biophysical properties of the NPC, transport receptors, and cargoes. We find that NPC components relevant for translocation are positively charged at physiological pH, while all analyzed transport receptors are negatively charged. Electrostatic interactions between a transport receptor and the NPC could result in an energy gain of a few kBT, which in turn could significantly increase the probability that a transport receptor enters the NPC. Together, our work suggests that charge complementarity between the NPC and transport receptors may be an important criterion for selective translocation through NPCs.

1296/B509

Rae1 Interacts with the Cohesin Subunit SMC1 at the Spindle Pole.

R. Wong[1,2], G. Blobel[2]; [1]Frontier Science Organization, Kanazawa University, Kanazawa, Japan, [2]Laboratory of Cell Biology, Rockefeller University, New York, NY

Accurate mitotic chromosome segregation depends on the formation of a microtubule-based bipolar spindle apparatus. We report that the cohesin subunit SMC1 (Structural Maintenance of Chromosomes subunit 1), is recruited to microtubule-bound Rae1 (RNA export factor 1, also a nucleoporin during interphase) at the mitotic spindle pole. We locate the Rae1 binding site to a 21 residue-long region, SMC1 947-967 and provide several lines of evidence that phosphorylation of Ser957 and Ser 966 of SMC1 stimulates binding to Rae1. Imbalances in these assembly pathways caused formation of multipolar spindles. Our data suggest that cohesin's known bundling function for chromatids in mitotic and interphase cells extends to microtubules at the spindle pole.

1297/B510

Nesprin 4: A Novel Nuclear Envelope Protein Induces Kinesin-Mediated Cell Polarization.

K. J. Roux[1], M. Crisp[1], Q. Liu[1], D. Kim[1], S. Kozlov[2], C. L. Stewart[3], B. Burke[1]; [1]Anatomy and Cell Biology, University of Florida, Gainesville, FL, [2]Center for Advanced Preclinical Research and Mouse Cancer Genetics Program, SAIC-Frederick, Inc., National Cancer Institute at Frederick, Frederick, MD, [3]Institute of Medical Biology, Immunos, Singapore

The LINC-complex couples the nucleoskeleton to the cytoskeleton through protein interactions that span the nuclear envelope. Interactions between Sun proteins and nesprins, constituents of the inner and outer nuclear membrane (INM and ONM), respectively, are fundamental to this complex. Thus far, three ONM nesprins have been found to interact with either cytoskeletal actin or intermediate filament-associated plectin. We have identified a novel member of the nesprin family, nesprin-4 (Nesp4), Similar to other nesprins, the localization of Nesp4 to the ONM depends on its interactions with INM Sun proteins. However, unlike the rather ubiquitous nesprins 1-3, expression of Nesp4 appears restricted to secretory epithelial cells in various exocrine tissues. The cytoplasmic domain of Nesp4 was found to associate with kinesin light chains and Kinesin-1. Furthermore, Nesp4 recruits kinesin light chain-1 (KLC1) to the nuclear envelope through the KLC1 cargo binding-domain. Expression of Nesp4 can induce a dramatic microtubule- and KLC1-dependent displacement of the nucleus from the centrosomes and associated Golgi complex. In light of these findings, we propose a role for Nesp4 in the establishment and maintenance of nuclear positioning in highly polarized secretory epithelial cells.

1298/B511

Identification and Analysis of Genes That Interact with MPS3.

J. M. Friederichs, E. S. Stensrud, S. Jaspersen; Stowers Institute for Medical Research, Kansas City, MO

SUN proteins (for Sad1-UNC84 homology) are a conserved family of inner nuclear membrane proteins characterized by a ~150 amino acid region of homology in their C-termini, known as the SUN domain. SUN proteins function as linker proteins, which bridge the inner and outer nuclear membranes to connect the nucleus with the cytoskeleton. Mps3 is the sole S. cerevisiae SUN protein. Mps3 is a structural component of the spindle pole body (SPB), the yeast centrosome-equivalent organelle, and as such, is essential for spindle assembly and chromosome segregation. However, a number of recent studies have suggested additional non-essential functions for Mps3, including establishment of sister chromatid cohesion, telomere tethering and gene inactivation. SUN proteins in other organisms likely share many of these Mps3 functions. To further investigate the diverse roles that SUN proteins play in the cell and identify possible Mps3 binding partners, we took advantage of the powerful genetic features of yeast and our large collection of mps3 mutants. Systematic synthetic genetic analysis (SGA) was performed on three conditional mps3 SUN domain mutants and one non-conditional N-terminal mps3 mutant allele (mps3Δ75-150). Overall, the genetic interactions that we identified in our screens showed enrichment for genes involved in microtubule dynamics and the spindle checkpoint, consistent with Mps3's known function in SPB duplication and spindle assembly. We also found that several classes of chromosome binding proteins, such as histone-modifying enzymes, kinetochore components and telomere binding proteins, frequently showed genetic interactions with certain mps3 mutants. Further analysis showed that Mps3 directly interacts with chromatin binding proteins and that these interactions are essential for proper Mps3 localization to the inner nuclear membrane. Lastly, we found that the conserved SUN-like protein SLP1 and a putative binding partner are essential for growth of all three mps3 SUN domain mutants but are not required for viability of mps3Δ75-150. This suggests that this new protein family is performing some partially redundant function with the SUN proteins in SPB duplication that we are currently investigating.

1299/B512

The GLFG Repeats Of Nup98 Participate in Two Distinct Types of Binding Interactions That are Unique from Those of FXFG and FG Repeats.

S. Xu, M. A. Powers; Department of Cell Biology, Emory University School of Medicine, Atlanta, GA

Nup98, the only GLFG nucleoporin in vertebrates, is a dynamic protein that shuttles between the NPC, cytoplasm and intranuclear bodies. The C-terminal domain of Nup98 contains a pore-targeting motif that binds to Nup96 and Nup88. However, in vivo this domain is inefficient and requires a synergistic contribution of the GLFG domain for efficient NPC targeting. We previously hypothesized that this occurs through many weak but cumulative interactions between the GLFG repeats and FG repeats of other nucleoporins. In myelogenous leukemias, the Nup98 gene can be translocated with one of several homeobox genes to form Nup98/homeobox fusions. Fusions always contain the Nup98 GLFG domain which is required for formation of intranuclear bodies. We hypothesized that these bodies assemble through interactions between FG repeats, as proposed to occur at the NPC. Through FRAP of Nup98/HoxA9 constructs containing varying numbers of FGs, we found that the dynamic association of Nup98/HoxA9 is indeed directly proportional to the number of FG repeats with a threshold of 21 repeats. Similarly, the efficiency of Nup98 targeting to the NPC was also proportional to the number of repeats Unexpectedly however, FG domains from other human nucleoporins were unable to substitute for the GLFG domain in NPC targeting or in formation of nuclear bodies. In contrast, a GLFG domain from the yeast Nup116p was fully functional. Thus, enhanced NPC targeting and assembly into bodies are characteristic of GLFG repeats. Interestingly, hexanediol, a reagent that disrupts the permeability barrier of the NPC, disperses both Nup98 and Nup98/HoxA9 intranuclear bodies but does not release Nup98 from the NPC. This suggests NPC targeting occurs through interaction of the GLFG domain with a non-FG repeat nucleoporin. In keeping with this, we found that Nup98 binds to Nup93. We predict that this interaction occurs through the GLFG domain of Nup98 and is responsible for the enhanced pore targeting. However, the sensitivity of GLFG nuclear bodies to hexanediol suggests that self-interaction of GLFG repeats, as occurs in the nuclear bodies, plays a significant role in the permeability barrier of the NPC. Experimental tests of this model will be discussed

1300/B513

Lamin B Receptor Mutants Induce Severe Morphologic Changes in Human Osteosarcoma and Glioblastoma Cells.

M. Zwerger[1], K. Richter[2], P. Lichter[2], H. Herrmann[1]; [1]B065 - 'Functional Cellbiology', DKFZ - German Cancer Research Center, Heidelberg, Germany, [2]B060 - 'Division of Molecular Genetics', DKFZ - German Cancer Research Center, Heidelberg, Germany

The human Lamin B receptor (LBR) is an integral membrane protein of the inner nuclear membrane (INM) and is essentially involved in binding of the lamina and chromatin to the nuclear envelope (NE). In addition, LBR binds DNA directly as well as indirectly via the heterochromatin protein 1 (HP1). Notably, LBR exhibits also sterol reductase activity. Mutations in LBR have been found to cause severe diseases when both alleles are affected (Pelger-Huët Anomaly and Greenberg skeletal dysplasia). In order to develop a cellular model for the pathomechanisms of these diseases, we have investigated the effect of LBR mutants on nuclear architecture in various cell lines by transfecting the corresponding YFP-fusion proteins. We found that overexpression of distinct LBR carboxyterminal truncations leads to a very severe phenotype in two cell lines, the osteosarcoma cell line U2OS and the glioblastoma cell line T98G, associated with dramatic alterations of nuclear size and shape accompanied by a massive compaction of chromatin. In addition, vacuole formation and - in later stages - the breakdown of the NE occurs, as demonstrated by electron microscopic analysis. We identified specific protein domains and amino acid residues responsible for this effect and found that the change of one amino acid is sufficient to induce this cascade of morphologic changes. However, other cell lines such as HeLa, MCF7 or SW13-F8 tolerated by and large the expression of LBR disease mutants. Furthermore, the overexpression of corresponding mutants of two authentic other sterole reductases, sterole reductase 1 (SR-1, TM7SF2 gene product) and sterole reductase 2 (SR-2, DHCR7 gene product), exhibited comparable affects in U2OS and T98G cells. Future experiments will address the question, if gene expression regulation is altered after expression of these mutants, and if corresponding gene expression programmes are affected in Pelger-Huët Anomaly and Greenberg skeletal dysplasia patients.

1301/B514

Lamin A Interacts with Nucleoporin Nup88: Implications in Human Aging?

Y. Lussi, I. Hügi, B. Fahrenkrog; M.E Mueller Institute for Structural Biology, Biozentrum, Basel, Switzerland

The nuclear intermediate filament protein lamin A is a constituent of the nuclear lamina, which is engaged in the organization of heterochromatin and nuclear architecture and is providing a platform for protein complex assembly. Mutations in the lamin A gene are associated with a diverse array of human disease, one of which is Hutchinson-Gilford progeria syndrome (HGPS), a rare disorder characterized by segmental premature aging. Distinct components of the nuclear lamina are interacting with nuclear pore complexes (NPCs), which are known to mediate all macromolecular trafficking between the nucleus and cytoplasm. Here, we have identified the nucleoporin Nup88 as novel interaction partner of lamin A by GST-pull down experiments. Furthermore, solution-binding assays revealed that it is the N-terminus of Nup88 that is binding to the tail domain of lamin A. The interaction was confirmed *in vivo* by immunoprecipitation assays in HeLa cells. Moreover, we found that overexpression of GFP-lamin A is masking the binding site of Nup88 antibodies in immunofluorescence assays, supporting the interaction of lamin A with Nup88 in a cellular context. Interestingly, a significant downregulation of Nup88 mRNA levels in old aged human cells and in cells derived from HGPS patients has been described, implicating Nup88 in human aging and supporting the link between lamin A and Nup88. In future, we will investigate if the interaction between lamin A and Nup88 is lost in HGPS cells and if this loss of interaction leads to age-related effects.

1302/B515

The C-terminal Domain of Nup98 Contributes to Bipolar Spindle Formation and is Regulated by Mitotic Phosphorylation.

M. Cross[1,2], M. A. Powers[1]; [1]Department of Cell Biology, Emory University, Atlanta, GA, [2]Biochemistry, Cell and Developmental Biology Graduate Program, Division of Biological and Biomedical Sciences, Atlanta, GA

The nuclear pore complex is comprised of approximately 30 different nucleoporins (Nups). During interphase of the cell cycle, this complex regulates trafficking in and out of the nucleus. However, during mitosis, the nuclear pore complex is disassembled and increasingly,

nucleoporins have been found to have alternate mitotic functions. We have utilized in vitro spindle assembly in Xenopus egg extracts to determine whether Nup98 plays a role during mitosis. When added to spindle assembly assays, the C-terminal domain of Nup98 stimulates uncontrolled growth of microtubules. The C-terminal domain similarly stimulates microtubule polymerization in both Ran-induced asters and preformed spindles. However, we have shown that excess microtubule growth does not require the known interaction between the Nup98 C-terminus and Nup96, a component of a nucleoporin complex associated with both the kinetochore and spindle. Instead, we have mapped the relevant region of Nup98 to a portion of the C-terminal domain without a previously characterized function. In support of a role for endogenous Nup98 in spindle assembly, addition of domain-specific antibodies or depletion of Nup98 from extract leads to formation of stable monopolar spindles. Importantly, addition of purified C-terminal domain to depleted extract rescues bipolar spindle formation. These findings strongly suggest that Nup98 itself, through a unique function of the C-terminal domain, is required for assembly of a proper mitotic spindle. We have observed co-localization of Nup98 and gamma tubulin in mitotic HeLa cells and several primary cell lines, suggesting that the site of function may be at the centrosome. We have also mapped interphase and mitotic phosphorylation within the C-terminal domain of Nup98. We find that mutation of S591, which blocks a mitotic phosphorylation site, significantly reduces the excess microtubule phenotype. Continuing experiments will elucidate the molecular mechanisms behind the observed phenotypes.

1303/B516

Lmo7, an Emerin-Binding Transcription Activator, Regulates C2C12 Myoblast Differentiation.

J. Holaska[1,2], K. M. Arfsten[1,2]; [1]Medicine/Cardiology, University of Chicago, Chicago, IL, [2]Institute for Cardiovascular Research, University of Chicago, Chicago, IL

X-linked Emery-Dreifuss muscular dystrophy (EDMD) is inherited through mutations in *EMD*, which encodes the inner nuclear membrane protein emerin. EDMD is characterized by progressive skeletal muscle weakening, contractures of major tendons and life-threatening irregular heart rhythms. We identified Lmo7 as a high-affinity emerin-binding protein that is specifically disrupted by the EDMD-causing P183H emerin mutation. Lmo7 regulates the expression of muscle regeneration genes implicated in EDMD. Further, mice containing a genomic deletion of Lmo7 have myopathic phenotypes. These observations suggest that Lmo7 is important for muscle regeneration. Muscle regeneration involves activation and differentiation of muscle stem cells (satellite cells) and subsequent fusion to and maturation of the myofiber. We monitored the differentiation of Lmo7-downregulated C2C12 myoblasts, a satellite cell-derived line, and found that Lmo7-downregulation significantly impairs myoblast differentiation. Interestingly, Lmo7 relocalizes from the nucleus to the cytoplasm during myoblast differentiation. Based on these observations we predicted that Lmo7 might regulate the expression of myoblast proliferation or early differentiation genes, or both. Supporting this hypothesis, Lmo7 downregulation results in decreased expression of myoblast proliferation and differentiation genes, including Pax3, Pax7, MyoD and Myf5. We also found that Lmo7 interacts with Pax3, MyoD and Myf5 promoters in C2C12 myoblasts and binds directly to the Pax3 and MyoD promoters in vitro. These observations suggest a model whereby Lmo7 directly regulates the expression of key myoblast proliferation genes by binding to their promoters and activating transcription. Because emerin-binding regulates Lmo7 activity, we propose that the X-EDMD phenotype might be caused, at least in part, by misregulation of key satellite cell proliferation and differentiation genes (Myf5, Pax3, MyoD), and subsequent failure of muscle regeneration.

1304/B517

The Lamin A mutation responsible for Hutchinson-Gilford Progeria Syndrome Leads to Loss of SUMOylation, Disruption of the Ran Protein Gradient, and Inhibition of NPC Basket Assembly.

J. Kelley[1,4], S. Datta[1,4], C. Yang[1,4], M. Matunis[3], V. Cordes[2], B. Paschal[1,4]; [1]Center for Cell Signaling, University of Virginia, Charlottesville, VA, [2]Max-Planck Institute for Biophysical Chemistry, Gottingen, Germany, [3]Biochemistry and Molecular Biology, Johns Hopkins University, Baltimore, MD, [4]Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA

The nuclear lamina is a mesh-like structure that provides a scaffold for nuclear envelope and chromatin attachment. Mutations in lamin A are responsible for multiple human diseases collectively known as laminopathies. Hutchinson-Gilford Progeria Syndrome is a premature aging disorder brought about by an internal deletion mutant of lamin A, referred to as Progerin. The process by which Progerin induced disruption of the nuclear lamina gives rise to premature aging is unknown. Here we report three novel defects in Progeria that have the potential to alter gene expression and function of the cell. Progeria fibroblasts display decreased SUMO 2/3 modifications and concomitant loss of the Ran protein gradient, which also correlates with reduced histone H3K9 trimethylation. Reducing SUMO 2/3 levels by expression of either a dominant negative Ubc9 or a SENP catalytic domain is sufficient to disrupt the Ran protein gradient. Thus, SUMOylation operates upstream of the Ran cycle and downstream of the nuclear lamina. Disruption of the Ran protein gradient is predicted to cause nuclear transport defects, and indeed, we find that a key component of the nuclear basket of the NPC, TPR, fails to undergo import in Progeria cells. This TPR import defect is recapitulated by NTF2 knockdown-mediated disruption of the Ran protein gradient. Additionally, Progerin expression inhibits import of a TPR NLS reporter protein, leading us to conclude that the TPR import receptor is sensitive to Ran nuclear levels, and that Ran system defects in Progeria cause TPR accumulation in the cytoplasm. Each of these Progeria phenotypes is reversible through treatment of the patient cells with a farnesyl transferase inhibitor (FTI). This indicates that the dominant negative effects of Progerin on these pathways requires its insertion into the nuclear membrane, and provides additional validation for FTI use in clinical treatment of Progeria. We propose that Progerin expression decreases SUMOylation of nuclear targets that regulate the Ran nucleotide cycle, leading to disruption of TPR import and assembly of the nuclear basket of the NPC.

1305/B518

Nuclear Localization of the SUMO E2 Ubc9 is Disrupted by the Lamin A Mutant Expressed in Hutchinson-Gilford Progeria Syndrome (HGPS).

S. Datta[1,2], J. Kelley[1,2], C. Yang[1,2], M. Matunis[4], V. Cordes[3], B. Paschal[1,2]; [1]Center for Cell Signaling, University of Virginia, Charlottesville, VA, [2]Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA, [3]Max-Planck Institute for Biophysical Chemistry, Gottingen, Germany, [4]Biochemistry and Molecular Biology, Johns Hopkins University, Baltimore, MD

We recently found that SUMOylation levels are reduced in primary fibroblasts from HGPS patients (Kelley et al. submitted). Ectopic expression of the HGPS lamin A mutant (termed Progerin) is also sufficient to reduce nuclear levels of SUMO2/3 in HeLa cells, suggesting lamina structure is critical for regulation of SUMOylation. Loss of SUMOylation in HGPS could be due to changes in the localization of the SUMOylation machinery; this includes a SUMO conjugating enzyme (E2) Ubc9 that localizes to both sides of the nuclear envelope. To determine whether the lamina regulates the distribution of Ubc9, we analyzed the localization of Flag-tagged Ubc9 following co-transfection with WT lamin A and Progerin. By IF microscopy, we observed that Progerin induced a cytoplasmic distribution of Flag-Ubc9. Using a Ubc9 antibody, we found that endogenous Ubc9 was excluded from the nuclei of cells expressing Progerin but not WT lamin A. Ubc9 displayed a punctate distribution along the nuclear rim in cells expressing either WT or Progerin forms of lamin A. These results suggest that Ubc9 targeting to the cytoplasmic side of the NPC (Nup358) still occurs, but that nuclear import is disrupted. Because the Ran protein gradient is disrupted in HGPS fibroblasts (Kelley et al.), we hypothesized that nuclear localization of Ubc9, known to occur by Imp13 (Mingot et al., 2001) is strictly dependent on a Ran protein gradient. While these results imply SUMOylation operates downstream of the Ran protein gradient, two results indicate that SUMOylation operates upstream of the Ran protein gradient. Expression of a dominant negative form of Ubc9 (C93S) or the catalytic domain of SUMO peptidase SENP2 is sufficient to reduce SUMOylation and disrupt the Ran protein gradient. We propose that disruption of the nuclear lamina initiates changes in chromatin structure that promote a reduction in nuclear SUMOylation; reduced SUMOylation disrupts the Ran gradient and causes a Ubc9 import defect. Because reduced Ubc9 import is predicted to exacerbate the reduction in nuclear SUMOylation initiated by the nuclear lamina, the lamina-SUMOylation-Ubc9 import pathway may represent a feed-forward mechanism that contributes to premature aging at the cellular level.

1306/B519

Nano-Apertures in a Plane Metal Film as an Optical Interface for Observing the Translocation through Single Nuclear Pore Complexes.

J. Wissler[1,3], M. Herrmann[2,3], D. Gradl[2,3], A. Naber[1,3]; [1]Institute of Applied Physics, Universität Karlsruhe (TH), Karlsruhe, Germany, [2]Zoological Institute II, Universität Karlsruhe (TH), Karlsruhe, Germany, [3]Center for Functional Nanostructures, Karlsruhe, Germany

Objective: We are investigating the diffusion of fluorescently labeled transport receptors (NTF2, Importin β) through the nuclear pore complex (NPC) using a large number of regularly arranged nano-aperture arrays (30x30 apertures per array, hole diameter ~150 nm). By combining near-field optics with Fluorescence Correlation Spectroscopy (FCS) we aim at contributing to the elucidation of the controversially discussed mechanism by which molecules pass through the nuclear pore complex. Methods and Results: The nano-apertures are fabricated in an opaque gold film by means of Focused Ion Beam (FIB) lithography. Nuclear membranes are manually extracted from *Xenopus laevis* oocytes and prepared on the structured gold surface under physiological conditions. Membrane patches spanning the nano-apertures are illuminated through the aperture using focused laser light in a conventional confocal microscopic set-up. If, by chance, a nuclear pore is located above an aperture, the translocation of fluorescence-labeled molecules can be observed by making use of the highly confined optical near-field of the nano-aperture. By applying FCS in such sub-wavelength confined excitation volumes [Levene et al., Science, 299, 681, 2003; Wenger et al., Biophys. J., 92, 513, 2007], specific single translocation activity (e.g. diffusion time, binding rate) shall be determined. Conclusion: It is possible to measure single molecule activity. To support data interpretation, electron microscopy is used after the optical experiment to map NPCs which might be located over the apertures.

1307/B520

Structural Basis for Extended Modularity of the Nuclear Pore Complex.

S. G. Brohawn, N. C. Leksa, E. D. Spear, T. U. Schwartz; Biology, MIT, Cambridge, MA

Transport of macromolecules across the nuclear envelope is exclusively mediated by the nuclear pore complex (NPC). In order to fully understand the transport process, knowledge of the NPC architecture is key. However, with 40-60 MDa in size the NPC is one of the largest assemblies in the cell thus making it a formidable challenge to characterize it structurally. Modularity within the NPC has been recognized for several years and can be exploited for structure determination following a divide-and-conquer approach. We have recently determined the crystal structure of the most abundant structural nuclear pore protein, Nic96. Together with the structure of a complex between two other scaffold proteins of the NPC, Nup107 and Nup133, we have now reanalyzed the phylogenetic relationships between the components of the NPC, with important conclusions for an Ur-NPC that preceded the extant form. This structure-based analysis allows us to substantially extend and specify the modularity concept of the NPC.

1308/B521

Nuclear Envelope Proteins NET25 and MAN1 Regulate Signaling in Myoblast Differentiation.

M. D. Huber, T. Guan, L. Gerace; Cell Biology, The Scripps Research Institute, La Jolla, CA

Certain mutations affecting nuclear envelope proteins cause muscular dystrophies, but the disease mechanisms remain elusive. The objective of this study was to determine the roles of Nuclear Envelope Transmembrane protein NET25 (LEM2) and its closely related paralog MAN1 in myogenic differentiation. Using an RNAi approach we show for the first time that both NET25 and MAN1 are required for myogenic differentiation of C2C12 myoblasts. NET25 depletion caused hyper-activation of ERK1/2 at the onset of differentiation, and pharmacological inhibition of the MAPK kinase MEK1/2 rescued myogenesis in NET25 depleted cultures. MAN1 depletion also inhibited myogenesis, but in this case inhibition of both MAPK and SMAD pathways was required to rescue differentiation. In addition, expression of silencing resistant human NET25 did not rescue myogenesis after MAN1 depletion. Thus, NET25 and MAN1 have at least partially non-redundant functions during myoblast differentiation. Our work links NET25 and MAN1 to myogenesis and supports the hypothesis that deregulation of cell signaling is a disease causing mechanism in muscular dystrophies.

1309/B522

Ran and Kap95 Function in Nuclear Pore Complex Assembly.

W. Shaikh[1], J. Spinks[1], M. Harville[1], K. Ryan[1,2]; [1]Biology, Texas A&M University, College Station, TX, [2]Program in Genetics, Texas A&M University, College Station, TX

Nuclear pore complexes (NPCs) are large protein structures that span the nuclear envelope (NE) and form the only passageways for molecules between the nucleus and the cytoplasm. Their biogenesis requires the coordinated assembly of hundreds of individual polypeptides to form the structures. In addition, this assembly must be restricted to the NE for proper nucleocytoplasmic transport. The GTPase Ran and Kap95/importinβ have previously been shown to be required for NPC assembly in both yeast and vertebrate systems. It has been well established that RanGTP binding to Kap95 disrupts Kap95's interactions with other proteins, thus freeing these proteins to carry out their functions. However, the factors operating downstream of the Ran-Kap switch during NPC assembly have not been fully defined. To address this question, we have screened a collection of *Saccharomyces cerevisiae* NPC assembly (*npa*) mutants for their ability to be suppressed by overexpression of *KAP95* or the Ran guanine nucleotide exchange factor *PRP20*. From this screen, we have identified non-allelic mutants whose growth defect is suppressed by overexpression of *KAP95* or *PRP20*. Additionally, at least one mutant appears to be rescued by either. As an independent approach to identify downstream factors, Kap95 and interacting proteins have been isolated from *prp20* mutant cells defective in NPC assembly. Because there is evidence to suggest that Ran and Kap95 are working at the cytoplasmic face of the NE, purifications of Kap95 have been done from both cytoplasmic and nuclear fractions and interesting differences are observed. Further characterization of these genetic and biochemical interactions will provide insight into the role of RanGTP and Kap95 during NPC assembly.

Chromatin & Chromosomes I (1310 – 1330)

1310/B523

Matefin/SUN-1, a Nuclear Envelope Protein, Is Essential for Embryonic and Germline Development and Homologous Pairing in Caenorhabditis Elegans Meiosis.

A. Fridkin[1], A. Penkner[2], E. Kaplan[1], V. Jantsch[2], Y. Gruenbaum[1]; [1]Genetics, Hebrew University of Jerusalem, Jerusalem, Israel, [2]Department of Chromosome Biology, Max F. Perutz Laboratories, University of Vienna, Vienna, Austria

The C. elegans mtf-1/sun-1 gene encodes matefin/SUN1, which is a nuclear membrane SUN-domain protein. Matefin/SUN1 has a germline-specific expression. Animals homozygous for mtf-1/sun-1 deletion allele show that matefin/SUN1 is essential for germline maturation and/or survival. Matefin/SUN1 is also required for early embryogenesis since mtf-1/sun-1 (RNAi) embryos die around the ~300-cell stage with defects in nuclear structure, DNA content and chromatin morphology. Previous studies (Malone et al, Cell, 2003) have shown that matefin/SUN-1 is required for centrosomes attachment to nucleus in the 2-cell embryos. Staining gonad of C. elegans hermaphrodite, homozygous for a gk199 deletion allele of mtf/sun-1 revealed that this is probably not the case at later stages of development. Recently a highly specific mutation (jf18) in mtf-1/sun-1 was identified. It provided direct evidence for active involvement of the nuclear envelope in homologous chromosome pairing in C. elegans meiosis. Our data show that mtf-1/sun-1 mediates a novel nuclear lamina pathway(s) required for embryonic development, for germline proliferation and survival and for homologous chromosome pairing.

1311/B524

Nuclear Envelope Transmembrane Proteins Influence Global Chromatin Organization.

P. Malik, N. Zuleger, V. Lazou, E. Schirmer; Institute of Cell Biology, School of Biological Sciences, University of Edinburgh, Edinburgh, United Kingdom

Genes at the nuclear periphery tend to be inactive and altering their positioning to the interior of nucleus results in their activation. Proteins of the nuclear envelope can recruit chromatin with specific epigenetic marks and can also recruit silencing factors that add new epigenetic modifications to chromatin sequestered at the periphery. The potential of the nuclear envelope (NE) in regulating gene expression is central to the favored hypotheses to explain the molecular mechanism underlying many NE diseases. To identify nuclear envelope transmembrane proteins (NETs) that could influence gene regulation, we are testing the effects of overexpression of NETs on global chromatin organization. We have tested principally novel NETs identified in a proteomics analysis of rodent liver nuclear envelopes for global affects on chromatin by over-expressing them in cell lines carrying different GFP-chromatin markers. Two of the new NETs cause global condensation of chromatin, particularly accumulating at the nuclear periphery. One NET further accumulates centromeres at the nuclear rim. Detailed analysis of this NET by live cell microscopy and FACS shows that it neither alters the cell cycle pattern nor arrests cells in any particular stage; however cells appear to fall out of cell cycle. Kinetic analysis and microscopy demonstrated that its overexpression leads to regular cell death, which is directly related to the levels of the protein expression. We expect that this is mediated by modification of chromatin that interacts with the nuclear envelope and are currently analysing by ChIP for changes in methylation, acetylation and phosphorylation, which are altered in the presence of this NET. That 24 other NETs including the well-characterized emerin, LBR and LAP2 had no effect on chromatin condensation serves as an internal control for the specificity of this NET function. Thus it appears that some of these novel NETs can have profound regulatory influence on chromatin architecture.

1312/B525

The Drosophila Melanogaster Fourth Chromosome: A Distinct Type of Heterochromatin.

S. Elgin, K. Huisinga, W. Leung, C. Shaffer, S. Wang, N. C. Riddle; Biology, Washington University, St. Louis, MO

The distal portion of the small fourth (dot) chromosome of Drosophila melanogaster is unusual in that it possesses many heterochromatic traits (including a high density of HP1), but nonetheless has a gene density comparable to euchromatic domains in the other chromosomes, ca. 80 genes in 1.2 Mb. In contrast to genes on the long chromosome arms, dot chromosome genes have larger introns, reflecting the higher density of repetitious elements on the dot (30% compared to 7%), and exhibit less codon bias. Mapping of the chromatin state using an hsp70-driven white gene reporter (mobilized by P element transposition) indicates that most of the dot chromosome is heterochromatic, as shown by a variegating eye phenotype. Those few sites showing euchromatic packaging (red eye phenotype) map to regions depleted for HP1. A recent screen to increase the number of such reporter lines (now 31 lines) confirmed that insertion adjacent to or within 10 kb of

repetitious element 1360 leads to variegation, but also demonstrated that the 1360 pattern is not sufficient to explain all results. The observation that reporters on the dot chromosome arm are sensitive to chromosome four dosage, while reporters at other locations are not, suggests that the dot heterochromatin has a unique make-up. Of the three H3K9 histone methyl transferases (HMTs) in Drosophila, only dSETDB1 appears to have a major role on the dot chromosome. Comparing the impact on loss of HMT activity, dSETDB1 depletion has the greatest effect on dot chromosome genes, while depletion of SU(VAR)3-9 has the greatest impact on regulation of transposable elements, comparing the number misregulated in each case. Use of GAL4-driven hairpin knock-down constructs demonstrates that dSETDB1 has a critical role in maintaining gene silencing during development. Interestingly, depletion of dSETDB1 alone is more deleterious than depletion of both dSETDB1 and SU(VAR)3-9, suggesting that balance in modification between the different heterochromatin compartments may be more important than the overall level of histone H3K9 methylation. Supported by NIH grants GM68388 and GM73190.

1313/B526

Distinct Chromatin Structure of Epigenetic Silencing Mutants in Yeast.

E. Y. Xu[1], K. Zawadzki[1], Y. Chong[2], B. Andrews[2], J. Broach[1]; [1]Molecular Biology, Princeton University, Princeton, NJ, [2]Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, Toronto, ON, Canada

The *Saccharomyces cerevisiae* mating-type genes are subject to epigenetic expression: these genes when resident at an expressor locus *MAT* are actively transcribed to establish the mating type of the cell, while the same genes resident at storage loci *HML* and *HMR* are completely repressed. Silencing of the mating-type genes at *HML* and *HMR* depends on the assembly of a repressive chromatin structure consisting of Sir2, Sir3, Sir4 and the core histones. This repressive chromatin structure can be established, maintained and stably inherited in mitotic and meiotic cells. To explore the mechanism of epigenetic silencing in detail, we have developed a fluorescent reporter construct by inserting GFP reporter genes into the *HML* silent locus and demonstrated that some partial silencing mutants, such as *sir1*, consist of two distinct populations, one repressed and one derepressed. Combining the technologies of FACS sorting and chromatin immunoprecipitation, we demonstrated that these two populations in *sir1* have different chromatin structure at the HM loci. The silent populations have intact chromatin structure and the active ones do not. To characterize the partial silencing mutants globally, we performed a genome-wide screen with the reporter construct. Using the high-content screen system and image analysis, we are able to identify additional partial silencing mutants. These studies reveal two types of partial silencing mutants - those involved in establishment or maintenance of the silent chromatin, which show epigenetic inheritance of silencing; and those involved in the integrity of silent chromatin, which exhibit “leaky” silencing.

1314/B527

A Recombination-Independent Hot Spot of Homologous Chromosome Pairing at the Sme2 Locus in Fission Yeast.

D. Ding[1], Y. Hiraoka[1,2]; [1]BioICT Group, Kobe Advanced Research Center, NICT, Kobe, Japan, [2]Graduate School of Frontier Biosciences, Osaka University, Osaka, Japan

Pairing of homologous chromosomes during meiotic prophase is achieved through two major steps: first, telomere clustering and chromosome movements spatially align homologous chromosomes side-by-side; and second, homologous recombination followed by the formation of the synaptonemal complex establishes physical connections between the homologous chromosomes. However, the mechanism of recognition between homologous chromosomes, in the context before double strand break (DSB) formation, remains largely unknown. In an attempt to search the mechanism responsible for recognition and pairing, we found a pairing hot spot at the *sme2* locus, which is located on the short arm of chromosome II. The *sme2* gene codes a non-coding RNA, the meiRNA, which is required for the Mei2-dot formation during meiosis. We found that the *sme2* locus shows higher pairing activity as compared with all the other chromosome loci examined, and this pairing activity remains at the wild type cell level in a DSB-deficient *rec12* mutant, indicating that this pairing activity is independent of DSB formation. Deletion of *sme2* gene from its authentic location decreased pairing activity at its proximal region, while translocation of *sme2* to the *lys1* locus on chromosome I or the *ade8* locus on the long arm of chromosome II elevated pairing activity at these sites. Furthermore, we found a high frequency of ectopic associations between two copies of *sme2* when they simultaneously present on different chromosomes. Our data imply that *sme2* loci may function as an active pairing site in recognition and pairing of homologous chromosome.

1315/B528

Fine-scale Dynamic Analysis of Bacterial Chromosomes.

S. C. Weber[1], J. Theriot[1,2]; [1]Biochemistry, Stanford University, Stanford, CA, [2]Microbiology and Immunology, Stanford University, Stanford, CA

The bacterial chromosome is packed inside a cell orders of magnitude smaller than its contour length, yet displays a remarkable degree of organization. Although the details vary from species to species, each chromosomal locus occupies a specific position in the cell that linearly corresponds to its location on the chromosome (Teleman et al 1998, Niki et al 2000, Viollier et al 2004, Nielsen et al 2006). The factors responsible for the ~1000-fold DNA compaction are not well understood. Nor is it known how the ordered arrangement of chromosomal loci is established and maintained. Here, we take both an experimental and a theoretical approach to probe the molecular and physical constraints on the bacterial chromosome. Single particle tracking of fluorescently-labeled loci in *Escherichia coli* and *Caulobacter crescentus* reveal sub-diffusive motion not only at the origins, as was seen for both chromosomes in *Vibrio cholerae* (Fiebig, Keren and Theriot 2004), but throughout the chromosome. Perturbations, including treatment with rifampin, chloramphenicol, A22 and novobiocin, suggest that entanglement of the DNA polymer and metabolic activity significantly contribute to chromosome mobility. Confinement by the cell wall appears to affect the scaling of mean square displacement with time interval, τ. Brownian dynamics simulations are being used to gain insight into experimental results.

1316/B529

Escherichia Coli Mutants Deficient in Nucleoid Proteins Exhibit Distinct Genome Folding.

R. L. Ohniwa[1], H. Muchaku[1], K. Morikawa[1], T. Ohta[1], S. Saito[1], C. Wada[3], K. Takeyasu[2]; [1]Graduate School of Comprehensive Human

Studies, University of Tsukuba, Tsukuba, Japan, [2]Gradutate School of Biostudies, Kyoto University, Kyoto, Japan, [3]Yoshida Biological Laboratory, Kyoto, Japan

The genomes of all living organisms are stored as flexible higher-order architectures. In bacteria, the genome DNA is packed in a cell as a form of "nucleoid". A set of distinct structural DNA binding proteins, such as Hu, H-NS, Fis etc., is believed to constitutively play major roles in genome packing, although little is understood on their contribution to nucleoid structures in vivo. Our previous study using atomic force microscopy has shown that, when Escherichia coli cells in the log phase were lysed, 30 nm fibers and 80 nm beads were released. In the present study, we explored the role of the nucleoid proteins in such structural-unit formation. Deletions of genes encoding Hu proteins (*hupA/hupB*) did not affect the structural units. However, deletions of IHF (*himA, himD*), Fis (*fis*), H-NS (*hns*), and Hfq (*hfq*) exhibited 10 nm fibers as the thinnest fiber unit. Namely, while the thinnest nucleoid unit in the wild-type cells was 30 nm fiber, the corresponding unit in *Δfis*, *Δhim*, *Δhns*, and *Δhfq* mutants was 10 nm fiber. RNase A treatment on these fibers gave rise to more 10 nm fibers released out of the cells. These results suggest that different nucleoid proteins and RNA differentially contribute to the 30 nm fiber and higher-order architectures.

1317/B530

Dynamic Behavior of Reconstituted Chromatin Fiber Observed by Fast-Scanning Atomic Force Microscopy.

Y. Suzuki, K. Hizume, M. Yokokawa, K. Takeyasu; Graduate School of Biostudies, Kyoto University, Kyoto, Japan

Chromatin function in living cells is closely related to changes in its structure. In this study, to obtain a visual glimpse of dynamic aspects of nucleosomes, we utilized a combination of techniques such as chromatin reconstitution and direct visualization of the chromatin fiber by fast-scanning atomic force microscopy (fast-scanning AFM). [Methods] Nucleosomes were reconstituted from purified Hela histones onto 1.8 kbp DNA template by a salt dialysis method. The reconstituted chromatin sample was deposited onto spermidine coated mica discs and imaged in buffer solution using the fast- scanning AFM, which uses a miniaturized cantilever and scan stage to reduce the mechanical response time of the cantilever and to prevent the onset of the resonant motion at high scan speed. The small cantilevers, with dimensions (L×W×H) of 10×2×0.1 μm, had resonant frequency in water of 600-1000 kHz and spring constants of 0.1-0.2 N/m. [Results and Conclusions] Although DNA construct used for reconstitution does not contain any nucleocsome-positioning signals, nucleosomes were efficiently formed on DNA fragment ([length of DNA used] / [number of nucleosome formed] □ 500bp). With fast-scanning AFM, the dynamic behavior of the reconstituted chromatin fiber was visualized under fluid in real time at 1-3 frames per sec. The core histone particle exhibited sliding motion along the DNA chain in a one-dimensional diffusion manner. This result supports the idea that chromatin fiber itself is also dynamical structure and nucleosome sliding is a possible mechanism to expose or occlude the recognition sites for the regulatory proteins. Our results also open the possibility that dynamics in biological reactions in a chromatin environment might be observed at the single-molecule level in sub-seconds.

1318/B531

An Insight into Nucleosome-Packing Mechanisms Obtained from Chromatin Reconstitution and Nano-Technology.

K. Hizume[1], T. Nakai[3], S. Araki[2], E. Prieto[1], K. Yoshikawa[2], K. Takeyasu[1]; [1]Biostudies, Kyoto University, Kyoto, Japan, [2]Graduate School of Science, Kyoto University, Kyoto, Japan, [3]Graduate School of Engineering, Tottori University, Tottori, Japan

All of the four core histones carry the amino-terminal tail regions, or "histone-tails", that are rich in lysine residues. Therefore, modification of thehistone-tails has been thought to be a significant factor for the higher-order arrangement of the nucleosomal array, since it is expected to contribute to the charge property of the histone-DNA interaction. In order to reveal the roles of histone tails in the formation of higher-order chromatin organizations, we examined the properties of reconstituted chromatin composed of tail-less histones and a long DNA (106-kb plasmid) template by using atomic force micrsoscopy. (1) Normal histone octamers form a "beads-on-a-string" nucleosomal array on the DNA at low-salt concentrations, and an aggregation at high-salt concentrations and/or with excess amount of core histones. (2) An addition of histone H1 to the beads-on-a-string promotes a formation of 30 nm chromatin fiber. (3)The tail-less histone octamers form a beads-on-a-string nucleosomal array, but the resultant nucleosomes did not aggregate at high-salt concentrations or with an excess amount of core histones. Analysis of the nucleosome distributions on DNA revealed that the attractive interaction between tail-less nucleosomes was weakened, and suggested that this was the major reason for the failure of 30 nm-fiber formation in the presence of histone H1. Thus, the attractive interaction between nucleosomes via histone-tails plays a critical role in the formation of the uniform 30-nm chromatin fiber. Namely, the positive charges of histone-tails are tightly correlated to the formation of H1-induced fibers. This is consistent with the fact that fission yeast has unique amino acid sequence in tail region of H2A and H2B and does not require linker histones for the maintenance of higher-order chromatin structures.

1319/B532

Statistical Mechanics and Kinetics of DNA Unwrapping in Nucleosomes.

M. M. Inamdar[6], T. Rimchala[7], H. G. Garcia[2], S. Privitera[2], J. Kondev[4], J. Widom[5], R. Phillips[1,3]; [1]Engineering, California Institute of Technology, Pasadena, CA, [2]Physics, California Institute of Technology, Pasadena, CA, [3]Kavli Nanoscience Institute, California Institute of Technology, Pasadena, CA, [4]Physics, Brandeis University, Waltham, MA, [5]Biochemistry, Northwestern University, Evanston, IL, [6]Indian Institute of Technology, Bombay, India, [7]Bioengineering, Massachusetts Institute of Technology, Cambridge, MA

The eukaryotic genome is organized into complex structures known as nucleosomes, which consist of DNA tightly wrapped around four pairs of proteins called histones. These structures raise questions about the accessibility of the wrapped DNA to proteins that must bind to the DNA for key processes such as transcription, replication and repair. Ingenious experiments in the recent past have suggested that nucleosomal DNA transiently unwraps from the histone core, exposing any protein target sites buried underneath. We present an equilibrium statistical mechanics model and a kinetics model for DNA unwrapping from a histone core. We use these models to make quantitative predictions for the equilibrium and dynamics of DNA accessibility as a function of burial depth.

1320/B533

Using Multi-Dimensional Proteomics to Define the Complete Protein Composition of Mitotic Chromosomes.

S. Ohta, F. Alves, L. Sennels, J. Rappsilber, W. Earnshaw; Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, United Kingdom

Mitotic chromosomes pose an intriguing problem for proteomic analysis, as they do not have a single defined protein composition. Upon nuclear envelope breakdown at prometaphase, numerous cytoplasmic and nuclear proteins associate with the chromosomes - some passively, and some in order to perform a variety of essential functions. We have used a multidimensional SILAC approach to develop a strategy for identification of structural proteins of chromosomes. An initial mass-spectrometry based proteomic analysis of chicken DT40 cell mitotic chromosomes identified 2,383 proteins in purified chromosomal fractions. Roughly 2000 of these are likely to be present in <30 copies per chromatid. Of the identified proteins, 110 had previously been annotated as centromere proteins and 13 as telomere proteins. This large number of centromere proteins was due to the fact that our analysis identified multiple variant forms of a number of the proteins. Our subsequent analysis utilized three classifiers in a combination of novel SILAC-based approaches to sort proteins into groups. These included the comparison of the abundance of proteins found on chromosomes versus in post-chromosomal supernatants; the exchange of proteins between chromosomes and cytosol; and the dependence of protein association with mitotic chromosomes upon the presence of a functional condensin complex. Analysis of these three qualifiers in three dimensions identifies a space containing of "true" chromosomal proteins, within which lie 49 novel uncharacterized proteins. Our preliminary studies of this group to date have identified five novel centromere proteins. This multi-dimensional profiling analysis can in principle be applied to any high-order structural complexes that can be enriched but for not purified to homogeneity.

1321/B534

Chromosome Segregation and Cohesin Dynamics in Arabidopsis Heterochromatin Mutants.

J. Ramahi, R. Maruthachalam, S. Chan; Plant Biology, UC Davis, Davis, CA

Proper chromosome segregation is maintained by a balance of chromatid cohesion and attachment of the mitotic spindle at the kinetochore. Centromeric heterochromatin has been implicated to be important for proper chromosome segregation in model organisms such as *S. pombe, D. melanogaster*, and several vertebrate systems. The specific role heterochromatin plays in proper chromosome dynamics has yet to be characterized outside of *S. pombe*, where heterochromatin disruption results in decreased loading of centromeric cohesion. *Arabidopsis thaliana* provides a model organism with orthologous heterochromatin modifications to vertebrate systems. Viable heterochromatin mutants allow us to understand the role that particular heterochromatic marks play in chromosome segregation and cohesin dynamics. A GFP marked version of the centromere specific H3 variant CENH3 localizes to centromeres, allowing us to detect additional centromere signals resulting from chromosome segregation errors. Chromosome dynamics will be assayed through an aneuploidy screen of *Arabidopsis* DNA methyltransferase mutants (*met1, cmt3 met1*), a histone modification mutant (*kyp*), and an RNAi mutant (*nrpd2a nrpd2b*). GFP-CEN3 has been crossed to heterochromatin mutants for analysis. We have developed a detailed assay for mitotic dynamics of centromeres, which will be used to analyze mitosis in Arabidopsis heterochromatin mutants. Deconvolution microscopy followed by Imaris analysis allows automated detection of GFP-CENH3 signals in 3D. We track centromere dynamics during congression to the metaphase plate, metaphase oscillations, and anaphase separation. To assay cohesin dynamics, we have generated a complementing SCC3-mCherry C-terminal fusion protein that rescues embryo lethality of an *scc3* null allele. SCC3-mCherry has shown to be loaded onto mitotic chromosomes, yet is not meiotically functional, resulting in sterile plants. Generation and characterization of N-terminal GFP- and mCherry- fusion proteins is underway for analyzing cohesin dynamics in heterochromatin mutants, as is developing an assay for cohesin dynamics *in vivo*.

1322/B535

Involvement of Smc5/6 Complex in Sister Chromatid Cohesion and Homologous Recombinational Repair in Vertebrate Cells.

A. Stephan, M. Kliszczak, H. Dodson, C. Morrison; National University of Ireland, Galway, Galway, Ireland

Smc5 and Smc6 are both essential in budding and fission yeast. Yeast Smc5/6 complex mutants are hypersensitive to IR, UV and MMS and Smc5/6 is recruited to HO-induced DSBs, facilitating intersister chromatid recombinational repair. Recent data have shown siRNA of human Smc5 or the non-Smc SUMO ligase, MMS21/ NSE2 to increase Rad51-dependent sister or intra-chromatid recombination, with gene targeting frequencies increased by siRNA of MMS21/ NSE2 or Smc5. ChIP revealed that Smc5/6 complex is recruited to (I-SceI-induced) DSBs and that Smc5/6 is necessary for cohesin loading at DSBs in human cells. To examine the role of the vertebrate Smc5/6 complex, we generated a conditionally-null Smc5 mutant line using the chicken DT40 model. Upon repression of Smc5, cells continued to proliferate normally, with no delay in the cell cycle. However, Smc5-deficient cells were sensitive to a wide range of DNA-damaging agents and showed elevated levels of chromosome aberrations upon irradiation. Formation of Rad51 foci after IR was delayed in Smc5 mutants and resolution of gamma-H2AX foci was attenuated in the absence of Smc5, suggesting defects in recombinational repair of DNA damage. Consistent with a marked defect in recombinational repair, gene targeting frequencies were greatly reduced in Smc5-null DT40 cells. After Smc5 repression, Nse2 levels declined greatly, indicating a destabilisation of the entire Smc5/6 complex. Smc2 condensin, Smc1 cohesin and Scc1 kleisin levels were unaffected. We then explored the impact of Smc5 deficiency on sister chromatid cohesion by measuring the inter-sister distances of Giemsa-stained chromosomes and of chromosomally-integrated Tet operator arrays. We found that the loss of Smc5 caused a consistent increase in the separation of the sister chromatids. A model for our findings is that Smc5/6 is involved in ensuring efficient recombinational repair of DNA damage by ensuring that sister chromatids remain together during the normal cell cycle. However, the impact on gene targeting also suggests that the Smc5/6 complex plays a more direct role in recombinational processes.

1323/B536

RNA Dependent Regulation of Mitotic Chromatin.

L. Strickland, A. Straight; Biochemistry, Stanford University, Stanford, CA

Eukaryotic cells must efficiently package their genome into the nucleus yet maintain its accessibility to the replication, transcription, and mitotic machinery. Epigenetic regulation of chromatin such as post-translational modification of histones and methylation of DNA are

thought to influence nuclear organization and gene expression but how these modifications alter chromosome structure is just beginning to be deciphered. Non-protein coding RNAs (ncRNA), typified by the Xist RNA that is required for X chromosome inactivation, are an important class of molecules that interact with chromatin and affect chromatin organization and gene expression. We have investigated the role of RNA in the organization and post-translational modification of mitotic chromatin. We isolated mitotic chromosomes from human tissue culture cells, digested the chromatin with RNases of varying specificity, and characterized changes in post-translational histone modifications after RNase digestion. Digestion of RNA:DNA hybrids with RNase H altered the distribution of histone H3 tri-methyl lysine-9 (3me-H3K9) on mitotic chromosomes. Pericentric H3K9 tri-methylation was re-distributed throughout chromosome arms after RNase H treatment suggesting that an RNA:DNA hybrid is important for the maintenance of pericentric heterochromatin. We have demonstrated that RNase H treatment releases an H3K9 histone methyltransferase (HMTase) activity from mitotic chromatin that can methylate both purified histone H3 and isolated mitotic chromosomes. We are currently using an RNAi-based screen of the known human HMTases and biochemical purification of the methyltransferase activity in order to identify the RNA:DNA hybrid dependent HMTase.

1324/B537

A Silencer in the Intervening Sequence of the Mouse Igk Gene Specifies Vk Gene Choice for Recombination.

Y. Xiang, W. T. Garrard; UT Southwestern Medical Center, Dallas, TX

Sis is a silencer within the intervening sequence between the joining (Jk) region and the most proximal Vk gene, which upon Vk-Jk joining is either deleted or inverted and far removed from the rearranged Vk gene destined to be expressed. Importantly, we have previously shown that this element is a recombination silencer responsible for recruiting Ikaros and targeting an Igk minilocus carried on a yeast artificial chromosome (YAC) to pericentromeric heterochromatin. We have now generated a deletion of Sis in the endogenous locus. Sis-/- mice exhibit normal levels of total Igk gene rearrangement and allelic exclusion. However, Sis-/- mice exhibit markedly decreased distal and enhanced proximal Vk gene usage for rearrangement. As a result, Sis-/- mice show a skewed Igk repertoire both in pre-B and splenic B cells. We conclude that Sis is a cis-acting element required for specifying the normal Igk antibody repertoire. Research supported by grants GM29935 and AI067906 from NIH and I-0823 from the Robert A. Welch Foundation.

1325/B538

Modulation of Intrachromosomal Homologous Recombination in Human Cells by BLM Helicase.

Y. Wang, B. C. Waldman, A. Waldman; Biological Sciences, University of South Carolina, Columbia, SC

Mutation of BLM helicase, a member of the RecQ family of DNA helicases, causes Blooms syndrome, an autosomal recessive disorder associated with aspects of premature aging, genome instability, and a strong predisposition to cancer. One hallmark of Bloom's syndrome is a greatly elevated level of sister chromatid exchanges. Studies of BLM, mostly conducted in vitro, have suggested that BLM directs homologous recombination (HR) events toward resolution in a noncrossover fashion and that BLM generally suppresses HR overall. We studied the influence of BLM on HR events occurring in the chromosomes of mitotically dividing mammalian cells. To do so, we used recombination substrate pLB4 which contains a thymidine kinase (tk)-neo fusion gene disrupted by the insertion of an oligonucleotide containing the 18 bp recognition site for endonuclease I-SceI. The disrupting oligonucleotide is located within the tk portion of the tk-neo gene. Also contained on pLB4 is a functional tk gene. We stably transfected normal human fibroblast cell line GM637 with a single copy of pLB4. Upon integration of pLB4, the functional tk gene and the disrupted tk-neo gene resided as closely linked direct repeats. A genomic double-strand break (DSB) was introduced within the integrated tk-neo gene by transient expression of I-SceI endonuclease. DSB repair events were recovered by selection for cells that gained resistance to G418 either via HR between the tk-neo fusion gene and the linked tk gene, or by nonhomologous end-joining (NHEJ). For HR repair, both crossover and noncrossover events were recoverable. When DSB repair was examined following transfection of siRNA directed against BLM expression, 89 out of 123 HR events recovered had resolved as crossovers. In controls, 43 out of 123 HR events were crossovers. This difference was highly statistically significant ($p = 5.8 \times 10^{-9}$ by a Fisher exact test). Our data also suggested that a greater portion of DSB repair may have occurred by HR (versus NHEJ) when BLM expression was knocked down. We believe that our work provides the first direct evidence that BLM deficiency may contribute to genome instability by promoting the resolution of bona fide HR events as crossovers.

1326/B539

Dynamic Reprogramming of Transcription Factors to and from the Subtelomere.

C. Mak[1], L. Pillus[1], T. Ideker[2]; [1]Biology, University of California, San Diego, La Jolla, CA, [2]Bioengineering, University of California, San Diego, La Jolla, CA

Transcription factors are most commonly thought of as proteins that regulate expression of specific genes, independently of the order of those genes along the chromosome. By screening genome-wide chromatin immunoprecipitation (chIP) profiles in yeast, we find that more than 10% of DNA-binding transcription factors concentrate at the subtelomeric regions near to chromosome ends. None of the proteins identified were previously implicated in regulation at telomeres, yet genomic and proteomic studies reveal that almost all have interactions with established telomere binding complexes. For many factors, the subtelomeric binding pattern is dynamic and undergoes flux toward or away from the telomere as physiological conditions shift. We find that subtelomeric binding is dependent on environmental conditions and correlates with the induction of gene expression in response to stress. Taken together, these results suggest that genome position effects involve not only co-expression of neighboring genes along a genome but have implications for the architecture and evolution of entire transcriptional regulatory networks.

1327/B540

Human PinX1 Mediates TRF1 Accumulation in Nucleolus and Enhances TRF1 Binding to Telomeres.

J. Yoo[1,2,3], B. Oh[4], Y. Park[1,2,3]; [1]Department of Pathology, Yonsei University College of Medicine, Seoul, South Korea, [2]Center for Chronic Metabolic Disease, Yonsei University College of Medicine, Seoul, South Korea, [3]Brain Korea 21 Project for Medical Science, Yonsei

University College of Medicine, Seoul, South Korea, [4]Cancer Metastasis Research Center, Yonsei University College of Medicine, Seoul, South Korea

Human PinX1 (hPinX1) is known to interact with telomere repeat binding factor 1 (TRF1) and telomerase. Here we report that hPinX1 regulates nucleolar accumulation and telomeric association of TRF1. In HeLa, HA-hPinX1 was co-localized with fibrillarin, a nucleolar protein, in 50% of the transfected cells and also resided in the nucleoplasm of the remaining 50%. Mutant analysis showed that the C-terminal region was important for nucleolar localization, while the N-terminus exhibited an inhibitory effect on nucleolar localization. Unlike HA- and Myc-hPinX1, GFP-hPinX1 resided predominantly in the nucleoli. Nuclear hPinX1 bound to telomeres and other repeat sequences as well, but nucleolar hPinX1 did not bind to telomeres, despite its interaction with TRF1. Nucleolar hPinX1 forced endogenous TRF1 accumulation in the nucleoli. Furthermore, TRF1 binding to telomeres was upregulated in cells overexpressing hPinX1. In an ALT cell line, WI-38 VA-13, TRF1 did not co-localize with hPinX1 in the nucleoli. In summary, hPinX1 likely interacts with telomere-unbound form of TRF1 in both the nucleolus and the nucleoplasm and leads to increased telomere binding of TRF1. The PinX1 function of mediating TRF1 nucleolar accumulation is absent in ALT cells, suggesting that it might be telomerase-dependent or might be modulated by TRF1 modification.

1328/B541

Telomere Length Regulation during Meiosis in Aspergillus Nidulans.

N. Wang, M. Vahedi, K. Kirk; Lake Forest College, Lake Forest, IL

In mammals, telomeres in germ cells have been found to be significantly longer than those in somatic cells. We are interested in developing a good model organism in a simple eukaryote to study this phenomenon. Our lab uses the filamentous fungus *Aspergillus nidulans* due to its exceptionally short telomeric repeat tracks of about 20 copies of a six base pair repeat observed in vegetative cells. The telomeres in sexual cells have not been studied. We hypothesize that in this fungus, the telomeres in the sexual spores are longer than those in the vegetative cells, analogous to what is seen in higher eukaryotes and some microbes. Two different stages of sexual spores will be studied: the ascospores inside mature cleistothecia and the meiotic cells from immature cleistothecia. A PCR based assay sensitive to small changes in telomere length is used to detect differences in the length of telomeres in the two kinds of sexual cells and the vegetative cells. Initial results from PCR, cloning, and sequencing are intriguing, and work is in progress to successfully extract DNA from the tough ascospores to study the telomere length in mature cleistothecia.

1329/B542

Regulation of R-loop-associated Recombination by DNA Topoisomerases.

Y. Yang, S. Huang, Y. Toh, T. Li; Department and Graduate Institute of Microbiology, College of Medicine, National Taiwan University, Taipei, Taiwan

R-loop, a structure composed of RNA/DNA hybrid and single-stranded DNA, and its associated DNA topological change occur during transcription. It is also known that both transcription and activation-induced cytidine deaminase (AID) are involved in recombination events around antibody diversification. However, the regulation of R-loop formation during transcription and its relationship with the action of AID remain unclear. In this report, AID was introduced into various Escherichia coli strains and levels of AID-stimulated antibiotic resistances/ mutagenesis (ASM) were examined to study factors in regulating formation of R-loop. The fact that the bacterial mismatch repair (MMR) system is required for ASM suggestes that AID exhibits similar functional activities and properties in bacterial and mammalian cells. Furthermore, we showed for the first time that R-loop is the targeting substrate for AID in vivo, as suggested by reduced ASM level in strains over-expressing RNase H, an enzyme distablizing R-loop via digestion of RNA in RNA/DNA hybrid. Topoisomerase I (TopA) and gyrase exhibited opposite activities in suppressing and promoting ASM/ formation of R-loop, respectively. Genetic analyses also revealed that recombination enzyme RecF, but not RecBC or RecE was critically involved in promoting ASM. Collectively, our results not only demonstrate the involvement of R-loop and topoisomerases in ASM, but also suggest a genetic model for screening factors involved in AID-mediated recombination, i.e. antibody diversity.

1330/B543

Evolutionary Dynamics of Gene Order and Heterochromatin in Malaria Mosquitoes.

I. V. Sharakhov[1], M. V. Sharakhova[1], A. Xia[1], I. V. Brusentsova[2]; [1]Entomology, Virginia Tech, Blacksburg, VA, [2]Institute of Cytology and Genetics, Novosibirsk, Russian Federation

The goal of this study was to determine the rates of inversion fixation and to identify molecular and cytogenetic features that correlate with unequal rates of karyotype evolution. We mapped 326 Anopheles stephensi, A. funestus, and A. gambiae cDNA and BAC clones to A. stephensi polytene chromosomes and used 231 uniquely located markers for comparative mapping with A. gambiae and A. funestus. Primary mouse antibodies against D. melanogaster HP1 and Lamin Dm0 were used for immunostaining the A. gambiae and A. stephensi polytene chromosomes. The comparative mapping has clearly demonstrated a striking contrast among chromosome arms in length of conserved segments. There are small conserved blocks (< 2 Mb) on arm 2R and large conserved blocks (up to 8 Mb) on 3R and 3L of An. gambaie. The analysis using the Genome Rearrangements In Man and Mouse (GRIMM) program revealed that the X chromosome has the highest rate of inversion fixation whereas autosomes vary in the inversion density: 2R>2L>3R>3L. The analysis of the An. gambaie genome revealed a significant negative correlation between the number of fixed inversions and the density of Matrix/Scaffold Attachment Regions (M/SARs) suggesting a role of nuclear architecture in determining the chromosome specificity of rearrangement rates. In addition, we found a positive correlation between the rates of inversion fixations and the simple repeat content on five chromosomal arms. This suggests involvement of simple DNA repeats in facilitating rearrangements. We found that a hotspot for inversion breakpoints corresponds to an intercalary α-heterochromatin. Detailed analysis of the chromatin morphology demonstrated that some euchromatic regions and all regions of β-heterochromatin have HP1 and Lamin. No antibody was detected in condensed α-heterochromatin. Our comparative genomic and cytogenetic studies of A. gambiae, A. funestus, and A. stephensi suggest that α-heterochromatin is responsible for chromosome breakage and β-heterochromatin is responsible for binding chromosomes to the nuclear envelope.

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1331/B544

Distinct Epigenetic Signatures at the Nuclear Periphery of Embryonic Stem Cells.

L. Luo[1], K. L. Gassman[1], L. Petell[1], C. L. Wilson[1,2], J. Bewersdorf[1], L. S. Shopland[1]; [1]The Jackson Laboratory, Bar Harbor, ME, [2]Department of Mathematics, University of Maine, Orono, ME

The epigenetic state of chromatin in embryonic stem (ES) cells is a key regulator of pluripotency. Various epigenetic marks have been mapped in the ES cell genome, providing important clues to the mechanisms of epigenetic gene control. However, additional "epigenetic" control of the pluripotent state may be mediated by the three-dimensional (3D) organization of the genome in the ES cell nucleus, since the location of genes in the nucleus can impact gene expression. To determine how genes of different epigenetic states are organized within ES cell nuclei, we measured their 3D distributions using quantitative confocal image analysis. We focused on the distributions of specific covalent histone modifications relative to the nuclear periphery, a compartment that accumulates facultative heterochromatin in differentiated cells and is thought to cause gene silencing. We find, however, that active loci localize to this compartment in undifferentiated mouse ES cells. Indeed, the ES cell nuclear periphery contains ~10% of the transcriptionally active genes, as marked by trimethylated lysine 4 or lysine 36 of histone H3 (H3-K4Me3 and H3-K36Me3). These findings are consistent with the locations of active transcription sites labeled by brief bromo-uridine incorporation. Surprisingly, the distribution of active genes is not unique to ES cells, but is similar in adult neuronal stem/progenitor cells (NSCs) and NIH-3T3 fibroblasts. In contrast to the nuclear distribution of active genes, repressed genes marked by trimethylated lysine 27 of H3 (H3-K27Me3) are more enriched at the periphery of ES cell nuclei. Further, H3-K27Me3 positions vary among different cell types, including NSCs, suggesting that pluripotent and lineage committed stem cells can be distinguished by the nuclear distribution of this repressive epigenetic mark. Together, these data indicate that the nuclear periphery of multiple mammalian cell types is compatible with both gene transcription and repression. Further, the nuclear peripheries of different cell types, including ES cells, contain distinct balances of active and repressed genes, implicating the nuclear periphery in the control of genome epigenetic states.

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DNA Architecture and Transcriptional Regulation: Exploring DNA's Mechanical Code.

H. Garcia[1], R. Phillips[2]; [1]Physics, California Institute of Technology, Pasadena, CA, [2]Applied Physics, California Institute of Technology, Pasadena, CA

DNA architecture plays a key role in determining spatial and temporal patterns of gene expression. This architecture encompasses both the nucleotide sequence (i.e., the information content) and the physical state of the DNA such as its spatial organization and mechanical properties. We explore transcriptional regulation by DNA looping in the lac operon as a concrete quantitative example, where transcriptional control is realized by the simultaneous binding of Lac repressor to two binding sites separated by hundreds of base pairs on the DNA. We develop a statistical mechanical model to quantify the in vivo energy cost of different DNA conformations in bacteria, which allows us to extract mechanical properties of DNA and to compare completely different regulatory systems such as the lac operon and the arabinose operon. By controlling DNA architectural properties such as the length of the intervening DNA and its sequence-dependent flexibility we generate a set of falsifiable predictions. We present our recent experimental efforts aimed at systematically probing those predictions at the single cell level.

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DNA Mechanics and Transcriptional Regulation in the lac Operon.

S. L. Johnson[1], L. Han[2], H. Garcia[3], J. F. Beausang[4], J. Widom[5], P. C. Nelson[4], R. Phillips[2]; [1]Department of Biochemistry and Molecular Biophysics, California Institute of Technology, Pasadena, CA, [2]Department of Applied Physics, California Institute of Technology, Pasadena, CA, [3]Department of Physics, California Institute of Technology, Pasadena, CA, [4]Department of Physics and Astronomy, University of Pennsylvania, Philadelphia, PA, [5]Department of Biochemistry, Molecular Biology, and Cell Biology, Northwestern University, Evanston, IL

The *E. coli lac* operon is a paradigm of basic transcriptional regulation in both prokaryotes and eukaryotes. But our understanding of this fundamental system remains incomplete, especially with regards to the action of the Lac repressor, which does not result in simply binary on/off transcriptional states. Rather repression occurs to different degrees depending on whether or not the repressor is allowed to bind simultaneously to two of the multiple operator sites present in the *lac* operon, thereby looping the operon's DNA, and what the probability of forming these loops is (Oehler *et al.* 1994, Müller *et al.* 1996). Recent work in our lab using the single-molecule technique of tethered particle motion (TPM) to study ~300 base-pair (bp) loops and sub-persistence length (<150 bp) loops has probed the effects of loop length and sequence on looping probability (Han *et al.*, unpublished manuscript). However, this work and the work of other labs has so far focused on observing looping between two operators, whereas in wild-type cells the operon contains three operators: O1, the strongest, which overlaps the promoter; O2, slightly weaker and located 401 bp downstream of O1; and O3, the weakest, located 92 bp upstream of O1. We are therefore building on our lab's previous work by using the TPM method to study looping in a wild-type, three-operator construct, and in operator knock-out constructs in which one of the operators has been removed. As expected, looping in the three-operator construct shows a higher degree of complexity than in comparable two-operator constructs. However, this complexity can be unraveled by the sequential elimination of each of the three operators, demonstrating that TPM can be used to study multi-loop constructs. A comparison of loop formation in the three operator construct to loop formation in operator knock-out constructs as well as in the artificial two-operator constructs our lab has already characterized will shed light on the effect of having three operators instead of two in the wild-type operon.

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Ultra-high Content Analysis of Estrogenic Activity by Automated Microscopy.

F. J. Ashcroft[1], E. D. Jones[1], M. Mancini[1], A. T. Szafran[1], I. Mikic[2], T. Moran[2], M. A. Mancini[1]; [1]Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, [2]Accelyrs, San Diego, CA

Estrogen receptor-α (ER) function is clearly linked to numerous developmental, reproductive and clinical conditions, being targeted by various endocrine disruptors. While reductionistic biochemical approaches have provided invaluable insight into many mechanistic details of gene regulation, a fully integrated systems biology-level appreciation at the single cell level is lacking. This is largely due to technical roadblocks in data acquisition and processing. We have previously described a single cell model of ER function utilizing an engineered HeLa cell harboring a multicopy integration of an ER-regulated prolactin promoter driven transcriptional reporter gene (PRL-HeLa; Sharp et al, 2006). Transient transfection with GFP-ER results in formation of discrete and dynamic fluorescent subnuclear foci that marks the prolactin promoter array (PRL-array). We report here the generation of a PRL-HeLa subclone stably expressing GFP-ER and development of a suite of image analysis and reporting tools that facilitate automated quantification of a wide range of PRL-array characteristics. Pipeline Pilot (Accelrys) was used to quantify PRL-array occupancy, size, and RNA polymerase II (Pol II) occupancy for a small panel of clinical and environmental compounds. In the absence of ligand, the PRL-array is unoccupied by ER; however, this result was highly dependent upon use of multi-well plates free of "estrogenic plastics/adhesives." Treatment with estradiol (E2, agonist) leads to a rapid recruitment of ER and Pol II, and array decondensation; alternatively, tamoxifen (antagonist) induces recruitment of ER, but not Pol II. Data from bisphenol A, DTT, octylphenol and other environmental compounds closely mimicked E2, and demonstrate the utility of automated imaging and analysis tools for high throughput screening of endocrine disruptors affecting ER. Importantly, the PRL-HeLa system is also amendable to study with ERb and ER-chimeras, and provides a panel of single cell models for use in intensive, ultra-high content screening opportunities for study of nuclear receptor functions.

1335/B548

Development of a Conventional Assay System for HCV Replication Using RNA Polymerase I-Dependent Expression Plasmid.

T. Yoshida, M. Ojima, M. Kondoh, K. Yagi; Osaka University, Osaka, Japan

Objective: Hepatitis C virus (HCV) is a cause of chronic hepatitis, cirrhosis, and liver cancer. There are ~170 million individuals infected with HCV in the world. The available therapy, interferon and ribavirin, has limited efficacy and severe side effects. Replication step of HCV is a promising target for anti-HCV therapy, but there are a few conventional screening systems for HCV replication. Transfection of the cells with in vitro synthesized HCV-related RNA genomes (7-9.6 kb) has been widely used as the assay for HCV replication. In the present study, we developed a plasmid-based assay system for HCV replication using RNA polymerase (pol) I-mediated gene expression system and luciferase as a reporter gene. Methods: We constructed an RNA pol I-dependent plasmid system using human RNA pol I promoter and mouse RNA pol I terminator. HCV reporter construct was prepared by replacing the HCV structural genes with luciferase in HCV1b RNA genome. The HCV reporter construct was inserted into the RNA pol I vector. Huh7 cells were transduced with the RNA pol I vector containing HCV reporter construct, followed by measurement of the luciferase activity. Results: To confirm the RNA pol I-mediated gene expression, we firstly prepared the RNA pol I vector containing luciferase gene and confirmed the luciferase expression in Huh7 cells. Next, we constructed the RNA pol I plasmid containing the HCV reporter construct. Transfection of the cells with the HCV replicon plasmid showed expression of luciferase. The luciferase expression was attenuated by treatment with an anti-HCV agent, interferon. These data suggest that the RNA pol I plasmid is useful for screening of anti-HCV agents. Conclusion: We developed the RNA pol I-dependent plasmid assay system for HCV replication, and this system will contribute to future pharmaceutical sciences for development of anti-HCV agents.

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Mapping Tissue-Specific Regulatory Elements of the CFTR Locus Using DNase-chip.

C. J. Ott[1], G. E. Crawford[2], A. Harris[1]; [1]Human Molecular Genetics Program - Children's Memorial Research Center, Northwestern University Feinberg School of Medicine, Chicago, IL, [2]Institute for Genome Sciences & Policy, Duke University, Durham, NC

Genes that display complex spatial and temporal transcriptional regulation often contain cis-regulatory elements that lie outside of the basal promoter. Often these elements exist within DNase I hypersensitive sites (DHS), regions of chromatin that are nucleosome-depleted and thus susceptible to nuclease digestion. Our objective was to locate tissue-specific DHS within the cystic fibrosis transmembrane conductance regulator gene (CFTR) locus, an important disease-associated gene that is expressed in the epithelia of several tissue types including lung, pancreas, and small intestine. We have utilized Nimblegen tiled microarrays to detect DHS at a high resolution along the locus (DNase-chip). The novelty of this work is its application to primary cells from certain epithelia that express CFTR and are relevant to CF disease. Chromatin was extracted from DNase I-treated nuclei and biotinylated linkers were used to extract DNased ends. Captured DNA was amplified by LM-PCR, labeled, and hybridized to tiled arrays. Tissue-specific DHS were mapped in primary human tracheal epithelial cells, primary human bronchial epithelial cells, the human bronchial epithelial cell lines 16HBE14o- and Beas2B, the human colon carcinoma cell lines Caco2 and HT29, and primary human skin fibroblasts (that do not express CFTR). Each cell type analyzed displayed a unique pattern of DHS along the locus. Interestingly, the lung cells that were studied contained a very different pattern of DHS than other CFTR-expressing cell types. This is of relevance since CFTR expression levels are significantly lower in airway epithelia than in digestive epithelia and may involve different regulatory mechanisms. Within our DHS we have identified several CpG islands, and binding motifs for tissue-specific and general transcription factors including HNF1, FOX factors, and C/EBP. Our data have identified potential tissue-specific cis-regulatory elements within the CFTR gene locus and highlights the different structural characteristics of the CFTR locus between cell types that express the gene.

1337/B550

Protein Expression Analysis of the [PSI+] and [psi-] Yeast Strains Using 2-D Gel Electrophoresis.

S. Syafruddin[1], S. Prosa[2], S. Zimmerman[1], M. Barlow[1], T. Kim[3], I. Evans[1]; [1]Biological Sciences, Rochester Institute of Technology, Rochester, NY, [2]Medical Sciences, Rochester Institute of Technology, Rochester, NY, [3]Chemistry, Rochester Institute of Technology, Rochester, NY

The purpose of these experiments is to determine whether there are significant differences in protein expression between two isogenic yeast strains, one of which contains a protein in its "prion" conformation. In previous projects DNA microarrays and Quantitative Real Time Polymerase Chain Reaction (QRT-PCR) were used to study changes in gene expression between two yeast strains, [PSI+] and [psi-]. The [PSI+] strain contains the prion form of the SUP35 protein, and this protein exists in a different conformation compared to the normal SUP35 protein. The SUP35 protein serves as the elongation release factor (eRF3) which functions to terminate the newly synthesized polypeptide chain. Since the protein is expressed in a different conformation (prion conformation) in the [PSI+] strain, the translation termination is no longer functional or successful and the read-through of stop codons occurs. The purpose of doing the DNA microarray was to determine whether the protein conformational change in the [PSI+] strain has any significant effects and causes differences in the gene expressions between these two strains. Over 100 genes were found to be over or under-expressed in [PSI+] when compared to [psi-]. The central dogma of biology is DNA -> RNA -> protein. Hence, if there are differences in the gene expression between these [PSI+] and [psi-] strains, there should also be disparity in the proteins produced by these strains. In order to detect protein differences, 2-dimensional gel electrophoresis is used to analyze the protein harvested from both strains. In the first dimension or isoelectric focusing (IEF), the proteins are separated according to their isoelectric points (PI). After being separated according to their PI, the proteins are then separated according to their molecular weight in the second dimension. Protein differences have been found between the [PSI+] and [psi-] yeast strains. We are identifying these protein changes in order to determine whether the yeast mRNA changes found previously translate into similar protein expression changes.

1338/B551

Transcriptional Profiling Studies of Isogenic Yeast Prion Strains.

S. Zimmerman[1], S. Syafruddin[1], Y. Tra[2], I. Evans[1]; [1]Biology, Rochester Institute of Technology, Rochester, NY, [2]Mathematics and Statistics, Rochester Institute of Technology, Rochester, NY

The objective of this study is to determine whether the presence of the Sup35 gene product in its "prion" conformation in the yeast strain [PSI+] causes changes in gene transcription. In the model organism Saccharomyces cerevisiae (yeast), the gene SUP35 codes for a translational release factor protein eRF3. This protein has been the subject of intense study as a model for prion proteins in eukaryotic organisms. A prion protein is a protein that is capable of assuming an alternate conformation distinct from that of its known functional conformation and in this altered "prion" conformation, the protein can have a different functional activity. eRF3, in its prion conformation, has the ability to induce "normal" eRF3 to convert to a prion conformation. In mammals this can cause neurodegenerative diseases called transmissible spongiform encephalopathies. However, in yeast this prion system causes no known pathology and may even give an evolutionary advantage. This is because the prion conformation of eRF3 is inefficient at translational release causing stop codon read through and alternate protein production. The variability in proteins produced may have a positive effect allowing the yeast to survive in diverse environments. We have previously done transcriptional profiling of the [PSI+] and [psi-] yeast strains. These results suggested there were over a hundred gene expression changes in the [PSI+] prion containing strain. In order to ensure that our [PSI+] and [psi-] strains are isogenic, our lab has cured or reverted the [PSI+] strain back to [psi-] by growing the strain on YPD plates containing guanidine hydrochloride. Further microarray studies on these isogenic strains have been done to confirm our earlier findings. The changes in gene expression are being characterized using GenMAPP to determine which biological pathways the over- and under expressed genes are involved in. The changes in gene expression that were found may have important implications for explaining the widely varied phenotypes for growth, viability, and survival in different environments found when [PSI+] and [psi-] strains of yeast are compared.

1339/B552

Finishing the Sequence of the Bombyx mori Genome.

K. Mita[1], Q. Xia[2]; [1]Division of Insect Sciences, National Institute of Agrobiological Sciences, Tsukuba, Japan, [2]Southwest University, Chongqing, China

We present a complete silkworm genome sequence obtained by the integration of data from two WGS projects performed independently in 2004. In the new assembly, sequence coverage of the merged WGS reads, together with a newly generated fosmid- and BAC-end data, reached 8.48x. The new assembly has the best continuity (> 3.7 Mb in N50 scaffold size, and 15.5 Kb in N50 contig, respectively) among the sequenced insect genomes. Using a high-density SNP linkage map consisting of 1,577 markers, about 87.4% of the scaffold sequences were anchored to all the 28 chromosomes. Comparison between the linkage map and the assembly indicated that both the assembly and the genetic map were reliable for subsequent analyses. The coverage of repeats in the Bombyx genome, 44%, is substantially higher than those in other insects, contributing larger genome size of *Bombyx*. De novo gene prediction was performed using gene finder BGF by pre-filtering classifiable TEs. It gave a gene count of 16,329. In conclusion, the new genome assembly made available a more precise annotation of the structure and function of the *B. mori* genome. The data presented and analyzed herein can facilitate us to explore evolutionary patterns of insect genomes and will serve as a useful tool for gleaning new insight into genetic, developmental and regulatory events.

1340/B553

The Link between Pif1p and Rad27p in Saccharomyces cerevisiae during Chromosomal Replication.

D. Butts[1], K. Paeschke[2], V. A. Zakian[2]; [1]Biology, Morehouse College, Atlanta, GA, [2]Molecular Biology, Princeton University, Princeton, NJ

The PIF1 gene of Saccharomyces cerevisiae encodes a 5'-to-3' DNA helicase, which genetic studies have shown to be involved in the maintenance of mitochondrial, ribosomal, and telomeric DNA and is also believed to play a role in chromosomal replication. Although Pif1 DNA helicase is evolutionarily conserved from yeast to human, the molecular function during replication remains to be clarified. Here we investigate whether Pif1 DNA helicase in yeast genetically interacts with another component of the replication machinery, Rad27p. The yeast RAD27 gene encodes a 5'-3' flap exo/endonuclease, which plays an important role in DNA replication for Okazaki fragment maturation. Genetic analyses indicate that Rad27p is not essential for cell growth except at high temperatures; however, rad27Δ mutants are extremely temperature sensitive and grow slowly. Currently we are testing whether or not double mutant rad27Δ pif1Δ cells are lethal or viable under various temperatures conditions. Here we show overexpression of Pif1p did not enhance cell survival in rad27Δ mutants, rather the cells

entered senescence at a faster rate. Although, we initially proposed that Pif1p serves as a bypass mechanism for the absence of Rad27p during replication, these genetic analyses indicate otherwise.

1341/B554

SIRT1 Regulates the Activity of Heat Shock Factor HSF1.

S. Westerheide[1], J. Anckar[2], S. Stevens[3], L. Sistonen[2], R. Morimoto[1]; [1]Biochemistry, Northwestern University, Evanston, IL, [2]Biology, Turku Center for Biotechnology, Turku, Finland, [3]Protein Chemistry Core Facility, University of Florida, Gainesville, FL

The transcription factor HSF1 regulates cellular responses to stress and aging. We show here that HSF1 is an acetylated protein, and that SIRT1 affects HSF1 acetylation levels. The kinetics of HSF1 acetylation correlate with the attenuation phase of the HSF1 activation cycle, suggesting a role for acetylation in downregulating the heat shock response. Mass spectrometry analysis shows that HSF1 is acetylated on at least 9 lysines, one of which is in the DNA binding domain and is conserved across all organisms including yeast. Mutation of this lysine, K80, causes a defective heat shock response when put back into HSF1 null mouse embryonic fibroblast cells. In human tissue culture cells, mutation of K80 inhibits the formation of stress granules, a hallmark of HSF1 activation. Further analysis shows that mutation of this lysine prevents DNA binding to the heat shock element. Using molecular modeling, we provide evidence that acetylation at K80 disrupts interactions with the DNA. We show that SIRT1 modulates acetylation at K80, and that SIRT1 regulates HSF1 occupancy on heat shock protein promoters. Therefore, we propose that SIRT1 activates HSF1 by keeping HSF1 in a deacetylated state that is competent to bind DNA, and that HSF1 subsequently becomes acetylated and loses DNA binding ability during the attenuation process. We conclude that SIRT1 is a central regulator of a network of stress-regulated transcription factors.

1342/B555

p53 Regulates TLR3 Expression and Function in Human Epithelial Cell Lines.

A. Eguma[1], M. Taura[1], M. Suico[1], T. Shuto[1], T. Koga[1], K. Komatsu[1], T. Komune[1], T. Sato[1], H. Saya[2], J. Li[3], H. Kai[1]; [1]Molecular Medicine, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan, [2]Gene Regulation, Advanced Medical Research, Keio University School of Medicine, Tokyo, Japan, [3]Microbiology and Immunology, University of Rochester Medical Center, Rochester, NY

Toll-like receptors (TLRs) are important sensors of microbial pathogens and mediators of innate immune responses. Although the signal transduction of TLRs is well elucidated, their basal regulation is largely unexplored. Here we show that the tumor suppressor p53 positively regulates the transcription of TLR3, a receptor for viral double-stranded RNA and poly I:C, by binding to the p53 site in the TLR3 promoter. TLR3 expression was lower in HCT116 $p53^{-/-}$ cells than in HCT116 $p53^{+/+}$ cells. Activation of p53 by 5-fluorouracil (5-FU) increased the TLR3 mRNA in epithelial cell lines with wild type p53 but not in cell lines harboring mutant p53. Knock-down of p53 by siRNA decreased the TLR3 expression. TLR3 mRNA was also lower in $p53^{-/-}$ mice liver and intestine than that in $p53^{+/+}$ mice. Furthermore, the poly I:C-induced phosphorylation of IκB-α, nuclear translocation of NF-κB, and phosphorylation of IRF-3, were drastically reduced in HCT116 $p53^{-/-}$ cells, indicating a dysregulation of the two signaling pathways governed by TLR3. Consequently, induction of IL-8 and IFN-β after poly I:C stimulation was impaired in HCT116 $p53^{-/-}$ cells. These results suggest that p53 influences TLR3 expression and function, and highlights a role of p53 in innate immune response in epithelial cells.

1343/B556

Structural Transitions of the RING1B C-terminal Region upon Binding the Polycomb cbox Domain.

R. Wang, U. Ilangovan, A. K. Robinson, V. Schirf, E. M. Lafer, B. Demeler, A. P. Hinck, C. A. Kim; Biochemistry, UT Health Science Center at SA, San Antonio, TX

Polycomb group (PcG) proteins play a critical role in maintaining cell identity and stem cell self-renewal. They function as multi-protein complexes and mediate gene repression via epigenetic marks on histones and the creation of repressive chromatin structures. RING1B and Polycomb (Pc) are two essential components of a multi-protein complex called Polycomb Repression Complex 1 (PRC1). These two proteins directly interact via the conserved C-terminal region of RING1B (C-RING1B) and the cbox domain of Pc. Our data shows that C-RING1B/cbox interaction displays a 1:1 stoichiometry with dissociation constants ranging from 9.2 nM to 180 nM for the different Pc orthologs. NMR analysis of C-RING1B alone and in complex with the cbox domain reveals a conformational tightening upon binding Pc cbox. This conformational change within C-RING1B may rise from the presence of two stretches of conserved sequences separated by a variable linker sequence. We have also solved the crystal structure of the C-RING1B/cbox complex to 1.8 Å. The structure reveals that the C-RING1B is able to bind to ~30 amino acid peptides that do not necessarily have high sequence identity but rather, possess the ability to extend a beta sheet region within C-RING1B. The complementary beta sheet region formed by sections from both proteins likely results in stabilization of the entire complex. Our results suggest that C-RING1B possesses inherent flexibility allowing C-RING1B to bind a variety of different proteins. While the same binding surface on C-RING1B is utilized, C-RING1B is able to accommodate different sequences. This conformational flexibility may allow RING1B to associate with different factors that recruit RING1B and other PcG proteins to different genomic loci.

1344/B557

Functional Analysis of Dd-grp94 (Dictyostelium discoideum-glucose Regulated Protein 94 Kilodalton) Gene using RNA Interference.

S. N. Baviskar, M. S. Shields; Department of Biological Sciences, Idaho State University, Pocatello, ID

The 94kDa glucose regulated protein (grp94) is one of the calcium binding chaperones of the endoplasmic reticulum. Overexpression of grp94 is recognized in several diseases like goiter, Alzheimer's, lung cancer, breast cancer, and immune disorders. However, its physiological role and mode of action is unknown. We have ascertained the biological roles of grp94 protein in *Dictyostelium discoideum* using RNA interference (RNAi). Since grp94 is calcium binding protein and an efficient calcium buffer we hypothesize that complete or partial elimination of Dd-grp94 would affect calcium binding cytoskeletal proteins thereby affecting *Dictyostelium* cell structure, growth and differentiation. We tried to completely eliminate the endogenous expression of the gene by electroporating Dd-grp94 gene disruption vector

into wild type cells. We did not get knock out mutants despite several successful electroporation experiments and so we conclude that Dd-grp94 might be indispensable for cell viability. Using RNAi, we down regulated Dd-grp94 protein and assayed Dd-grp94 RNAi mutants and parental cells for cell size, growth and differentiation. We found considerable reduction in cell size and growth rate in Dd-grp94 RNAi mutants as compared to parental cells. We also observed a marked relative delay in differentiation (formation of fruiting bodies from unicellular amoebae during starvation) in Dd-grp94 RNAi mutants following starvation. Gene replacement studies on several other genes of *Dictyostelium* have shown that their null mutants also exhibit reduced growth rate and reduced cell size like Dd-grp94 RNAi mutants suggesting that these proteins and Dd-grp94 have some overlapping functions. Since these proteins do not functionally compensate for down regulation of Dd-grp94 expression, we conclude that Dd-grp94 plays a crucial role in maintaining normal cell structure, growth and differentiation possibly through interaction with a variety of calcium binding cytoskeletal proteins.

1345/B558

The Transcription Factor CUX1 Physically and Functionally Interacts with the Promyelocytic Leukemia Zinc Finger (PLZF) Gene.

I. Fréchette, M. Darsigny, K. Brochu-Gaudreau, C. Jones, F. Boudreau; Anatomie et biologie cellulaire, Université de Sherbooke, Sherbrooke, QC, Canada

CUX1 is a transcriptional repressor of 200 kDa which is proteolytically processed during the S phase of the cell cycle to generate a shorter isoform of 110 kDa. In the gastro-intestinal system, CUX1 is expressed in the ileum and proximal colon of adult mice. It is well documented that CUX1 represses sucrase-isomaltase gene expression in colonic epithelium. However, little is known about the functional role of CUX1 in the colonic epithelium. We performed a microarray analysis utilizing CUX1 mutant mice and identified PLZF as being upregulated in the colonic epithelium of these mutant mice. Since PLZF is a transcriptional repressor implicated in the control of cell cycle, we undertook to characterize the interaction between CUX1 and the PLZF gene. First, a computer analysis of the PLZF gene predicted 21 potential interaction sites for CUX1. Cotransfection assays showed a decreased in the transcriptional activity of a 5'untranslated region of 3 kb (3 kb 5'-UTR) and of a 2kb portion containing the translation initiation site of the PLZF gene. CUX1 interacted with six sites in the 3 kb 5'-UTR portion and three sites in the 2 kb portion of the PLZF gene in gel shifts experiments. Binding of CUX1 on sites within the 2 kb portion of the PLZF gene was confirmed by competition assays. In vivo, CUX1 interacts with DNA-binding sites within the 3 kb 5'-UTR and the 2 kb portions of the PLZF gene as illustrated in chromatin immunoprecipitation experiment. Expression of CUX1 and PLZF proteins was next correlated in human colorectal cancer cell lines. A Western blot analysis showed weak expression of CUX1 in the majority of cancer cells and strong expression in normal colonocytes. In contrast, PLZF was induced in cancer cells but remained undetectable in normal colonocytes. Finally, overexpression of CUX1 in the Caco 2/15 cell line resulted in reduction of PLZF expression. Overall, these results suggest that PLZF is a direct target of CUX1 in the colonic epithelium. Since PLZF is fused to RARα in promyelocytic leukemia and CUX1 is expressed in uterine leiomyomas and in breast cancers, it will be of interest to investigate the implication of these two proteins during colorectal cancer.

1346/B559

Identification of Hypoxia-Regulated Genes in HIF-1α and β Dependent Manner.

H. Oh, S. Choi, H. Park; Life Science, University of Seoul, Seoul, South Korea

We investigated Hypoxia-Inducible Factor (HIF)-dependent changes in the expression of genes in response to hypoxia (0.1% Oxygen, 16 hr) by using HIF-1β defective BpRc1 cells and HIF-1α knock-down 3T3-L1 cells. This study identified 50 hypoxia-induced genes and 36 hypoxia-repressed genes by using cDNA microarray. Q-PCR analysis of nine genes confirmed our ability to accurately analyze changes in hypoxia-induced gene expression by microarray analysis. By comparing Q-PCR analyses of these nine genes in BpRc1 and hepa1c1c7 cells, we determined that eight of the nine hypoxia-induced genes are Arnt-dependent. Additional Q-PCR analyses of eight hypoxia-repressed genes confirmed, with a 50% probability, that microarray analysis was able to predicted hypoxia-repressed gene expression. Only two of the four confirmed genes were found to be repressed in an Arnt-dependent manner. Collectively 6 of these 13 genes (46.2% probability) showed a pattern of expression consistent with the microarray analysis with regard to Arnt-dependence. Finally, we investigated the HIF-1α dependence of these thirteen genes by Q-PCR analysis in HIF-1α knock-down 3T3-L1 cells. These analyses identified novel hypoxia-regulated genes and confirmed the role of Arnt and HIF-1α in regulating their expression. These results identify additional HIF target genes and provide a more complete understanding of hypoxia signaling. [This work was supported by a grant(R200706192003) from KOSEF. H Oh and S Choi are supported by BK21 program].

1347/B560

DDC Deregulates the Expression of Non-Coding RNA Which Is Prevented by Feeding Same.

J. Oliva, F. Bardag-Gorce, B. A. French, J. Li, S. W. French; Pathology, Harbor-UCLA Medical Center, Torrance, CA

Background: Mice fed DDC (0.1%) for 10 weeks, and then withdrawn from the drug for 1 to 4 months, reform Mallory-Denk Bodies (MDBs) when the drug is refed for 7 days. The liver cells that form MDBs increase in number and partially replace normal liver cells when the MDBs form. This is prevented by S-Adenosylmethionine (SAMe) when fed with the DDC. Method: Microarray analyses were done on RNA from livers of the mice refed DDC and DDC+SAMe. Real-time PCR was used to study the expression of non-coding RNA (ncRNA). Results: Microarray analysis of mice livers showed that MDB formation was associated with changes in the expression of a large number of genes. Microarray analysis showed that DDC induced the expression of H19 and antisense transcript (AIR) by 2 and 9 fold respectvely, and down regulated the expression of GTL2 by 8 fold. SAMe prevented this deregulation of the expression produced by DDC. The results were also measured using Quantitative RT-PCR for the drug fed and drug primed mice, which were refed the drug for 7 days. H19 and AIR were induced by 60 and 30 fold respectively, and GTL2 was reduced by 4 fold. SAMe prevented the deregulation induced by DDC. At this time MDB formation was observed. Conclusion: DDC is able to regulate the ncRNA expression of H19, Air and GTL2. When drug primed mice were refed DDC with S-adenosylmethionine (SAMe) (4g/kg body weight), the deregulation of these ncRNAs are prevented. The three ncRNAs found are imprinted genes, involved in many diseases such as hepatocarcinoma, renal carcinoma and neuroblastoma. The question remaining is: are the changes in the expression of ncRNAs involved in the mechanism of MDB formation? Supported by a grant NIH/NIAAA 8116 and Alcohol Center Grant on liver and pancreas P50-011999, morphology core.

1348/B561

Bisulfite Sequence Analysis of POU5F1, c-Myc, SOX2 and LIN 28 Following Bovine Somatic Cell Nuclear Transfer.

A. P. Davis, A. H. Bayles, C. Davies, E. W. Innes, Q. Meng, B. R. Sessions, K. L. White; Animal, Dairy and Veterinary Sciences and Center for Integrated Biosystems, Utah State University, Logan, UT

Aberrant methylation patterns have been proposed as a contributing factor in the poor development of embryos following somatic cell nuclear transfer (scNT), and could be a possible cause of the overall low success rate. The inability to epigenetically reprogram and erase somatic-specific methylation signatures found in the fibroblast donor cell line, specifically of transcription factors essential for proper early embryonic development, could explain the reprogramming deficiencies associated with scNT. The methylation pattern of four genes known to contribute to reprogramming of somatic cells to a pluripotent state were examined using the bovine model. POU5F1, c-Myc, SOX2 and LIN28 are transcription factors known to be actively involved in the development and differentiation of early embryos, as well as the maintenance of pluripotency, a necessary component of traditional reprogramming. Each gene was chosen because it contains either a CpG Island within the gene or within the promoter region of the gene making DNA methylation a plausible explanation for silencing in latter developmental stages. Regions within each CpG island of these four genes were examined by bisulfite sequencing using fibroblast donor cell lines, scNT blastocysts, and in vitro fertilized (IVF) blastocysts. The resulting sequences demonstrate that the methylation patterns of the scNT blastocysts show more similarity to the fibroblast donor cell line used for scNT as compared to the IVF blastocysts. These results indicate that the failed demethylation of key genes could be a cause of failed development following somatic cell nuclear transfer.

1349/B562

Dsk1p and Kic1p Complexes with SR Proteins and Cwf11p in Schizosaccharomyces Pombe.

M. A. Luca[1,2], J. Kang[1,2], A. Guven[2], Z. Tang[2]; [1]Scripps College, Claremont, CA, [2]Joint Science Department, The Claremont Colleges, Claremont, CA

Evolutionarily conserved SR (serine-arginine rich) proteins are important factors for alternative splicing and their activity is modulated by phosphorylation. We previously showed that fission yeast SR proteins, Srp1p and Srp2p, are the in vivo substrates of Dsk1p protein kinase. Kic1p has also been shown to be involved in pre-mRNA alternative splicing, and encodes an SR specific protein kinase. Cwf11p (complexed with Cdc5p) has been hypothesized to be part of a complex with Dsk1p and Kic1p, due to the role it plays in alternative splicing in S.pombe. By using S. pombe strains that overexpressed the SR proteins, the Dsk1p and Kic1p kinases, and the Cwf11 protein, the preliminary findings of a glycerol gradient support the involvement of the Dsk1p and Kic1p kinase complexes with the SR and Cwf11 proteins in alternative splicing. The initial findings using immunoprecipitation suggests that Cwf11p is involved in the Dsk1p and Kic1p kinase complexes. Our results also indicate that Srp1p and Srp2p are also part of the Dsk1p and Kic1p kinase complexes that may be involved in pre-mRNA splicing.

Epithelia II (1350 – 1363)

1350/B565

Second-hand Cigarette Smoke Inhibits Corneal Wound Healing by Stimulating Inflammation and Inhibiting Cornea Epithelial Cell Migration.

C. MA, M. Martins-Green; Cell Biology and Neurosciences, University of California, Riverside, Riverside, CA

It is known that environmental cigarette smoke causes delay in wound healing. However, the mechanism(s) involved in cigarette smoke-induced inhibition of healing are not known. Here we present results from studies on the effects of secondhand cigarette smoke on cornea wound healing. Fast cornea re-epithelialization and healing after injury is key in preventing damage to the underlying stroma. Therefore, identification of the processes affected by second hand smoke that affect corneal re-epithelialization, and their mechanisms of action, will provide insights into how to improve and accelerate cornea healing when people are exposed to this smoke. We used a cornea epithelium excision wound model and a cigarette smoking system that mimics second-hand cigarette smoking by humans, to evaluate the effect of second-hand cigarette smoke on cornea healing in vivo. We found that this smoke stimulates neutrophil-driven inflammation, and degradation of extracellular matrix molecules that are important for cell adhesion and migration. Localized and systemic depletion of neutrophils reversed in part the smoke-induced delay of cornea healing, suggesting that inflammation alone is not responsible for the smoke-induced delay of healing. One possibility is that smoke inhibits the function of molecules important in cell adhesion and migration. To test this possibility we used cornea epithelial cells in culture. Smoke inhibits the function of components of the focal adhesion complex, such as FAK and paxillin, and also of the cytoskeleton. The activation of RhoA, a member of the Rho-family of small GTPases, which are important regulators of cytoskeletal function, was also inhibited. In summary, our findings suggest that second-hand cigarette smoke inhibits cornea wound healing on the one hand by stimulating inflammation and on the other hand by stimulating extracellular matrix degradation and inhibiting activation of focal adhesion molecules, focal adhesion complex formation and Rho-GTPases, which in turn result in inhibition of cornea epithelial cell migration and wound closure.

1351/B566

Stimulation of Corneal Epithelial Migration by the PHSRN Peptide, Derived from Fibronectin, and it's Sequence Specificity.

A. Hattori[1,4], K. Hozumi[1], J. Ko[2], T. Chikama[3], K. Oomikawa[4], J. Kato[4], K. Ishida[4], M. Nomizu[1], T. Nishida[2]; [1]Laboratory of Clinical Biochemistry, Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan, [2]Department of Ophthalmology, Yamaguchi University Graduate School of Medicine, Ube, Japan, [3]Department of Ocular Pathophysiology, Yamaguchi University Graduate School of Medicine, Ube, Japan, [4]Research & Development Division, Biological Laboratory, Nitten Pharmaceutical Co., Ltd., Nagoya, Japan

[Objective] Fibronectin plays a critical role in diverse biological activities, including promotion of integrin-mediated cell adhesion, cell migration, and wound healing. Several active sites of fibronectin have been identified. Arg-Gly-Asp (RGD) sequence has been well characterized as an integrin-binding site. The Pro-His-Ser-Arg-Asn (PHSRN) sequence has been implicated as a synergistic site for the RGD.

Additionally, the PHSRN peptide induces cell invasion and accelerates wound healing. Here, we examined the bioactivity of PHSRN peptide on the corneal epithelial migration and its sequence specificity using various synthetic peptides. **[Methods]** The evaluation of corneal epithelial migration ex vivo was tested with an organ culture system of the rabbit cornea. Additionally, cell colony outgrowth-migration and cell chemotaxis using the Byden chamber in vitro was examined with a cell culture of human corneal epithelial (HCE) cells. **[Results and discussion]** The Ac-PHSRN-NH_2 peptide was a sufficient amino acid length for the corneal epithelial migration activity and the N-terminal acetyl and C-terminal amide groups contributed for the chemical stability in tear. Alanine substituted analyses of Ac-PHSRN-NH_2 peptide indicated that the Ser- and/or Arg-residues are important for promotion of the corneal epithelial migration and for stimulation of the HCE cell colony outgrowth-migration. The Ser-Arg motif is involving in various biologically active peptides, suggesting that the unique sequence interacts with cellular receptor(s) and regulates the biological functions. The Ac-PHSRN-NH_2 peptide may be useful as a therapeutic application including corneal wound healing.

1352/B567

The Imbalance between Anti-oxidant and Pro-oxidant Enzymes at the Ocular Surface of Dry Eye (Sjögrens Syndrome).

J. Cejkova[1], T. Ardan[1], C. Cejka[1,2]; [1]Department of Eye Histochemistry and Pharmacology, Institute of Experimental Medicine, Prague 4, Czech Republic, [2]Department of Ophthalmology for Children and Adults, 2nd Medical Faculty, Charles University, Prague 5, Czech Republic

Dry eye disease is a multifactorial disorder of the tear film and ocular surface that results in eye discomfort, visual disturbance, and often ocular surface damage. The factors leading to the tear film abnormalities may involve glandular inflammation, loss of hormonal support and autoimmune disease (Sjögrens syndrome, SS). Previously, oxidative reactions (lipid peroxidase levels and myeloperoxidase activity) in the tear film of patients suffering from dry eye were desribed. Purpose of this study was to detect enzymes contributing to reactive oxygen species generation at the ocular surface and also enzymes that cleave these toxic oxygen products. Therefore, the antioxidant enzymes (superoxide dismutase, catalase and glutathione peroxidase) and pro-oxidant enzymes (xanthine oxidoreductase/xanthine oxidase) were detected in the conjunctival epithelium of dry eye (SS) using immunohistochemical methods. Quantitative evaluation was obtained by means of image analysis. Conjunctival epithelial cells were obtained using the method of impression cytology and Millicell membranes. Normal eyes served as controls. As compared to the normal conjunctival epithelium, the pro-oxidant enzymes were highly expressed in the conjunctival epithelium of dry eye (SS), whereas the expression of antioxidant enzymes was reduced, particularly that of catalase and glutathione peroxidase. It is suggested that this imbalance between anti-oxidant and pro-oxidant enzymes contributes to the development of anterior eye surface oxidative injuries occurring in dry eye (SS).

1353/B568

Atrial Natriuretic Peptide and Retinal Pigment Epithelium Fluid Movement.

N. W. Baetz[1], W. D. Stamer[2], A. J. Yool[3]; [1]Cell Biology and Anatomy, University of Arizona, Tucson, AZ, [2]Ophthalmology and Vision Science, University of Arizona, Tucson, AZ, [3]Physiology, University of Adelaide, Adelaide, SA, Australia

Purpose: Atrial Natriuretic Peptide (ANP) mediated signaling regulates water movement in epithelial tissues throughout the body. The goal of this project was to determine the role of ANP in fluid transport across the retinal pigment epithelium (RPE) of the human eye. Methods: Primary human retinal pigment epithelial cells were cultured on 0.45µm Millicell nitrocellulose filters for 6-8 weeks according to established methods. The change in volume of media on the apical side of RPE monolayers was used to determine the direction and rate of fluid movement (µL/hr/cm^2) during 2 hour treatment periods with 4.5µM ANP at 37°C. Specificity of ANP effects was determined in the presence or absence of 45µM Anantin, a Natriuretic Peptide Receptor antagonist. Signaling through cGMP was characterized in separate water flux experiments using 2mM 8-Br-cGMP in the presence or absence of 2mM H-8, an inhibitor of Protein Kinase G (PKG). Results: Compared to the baseline flow rate (-0.58 + 2.65 µL/hr*cm^2; mean+SD), flow across ANP treated (12.60 + 2.11 µL/hr*cm^2) RPE monolayers increased significantly ($p = 0.02$, $n = 3$). Anantin (45µM) inhibited the ANP stimulated fluid transport (5.19 µL/hr*cm^2) (n=2). Compared to baseline volume flow (2.87 + 2.73 µL/hr*cm^2), flow across 8-Br-cGMP treated (21.35 + 1.59 µL/hr*cm^2) RPE monolayers increased significantly ($p<0.0005$, n=3). Application of H-8 did not significantly inhibit 8-Br-cGMP induced fluid movement (19.69 + 6.77 µL/hr*cm^2) ($p = 0.56$, n=3). Conclusions: Atrial Natriuretic Peptide stimulates fluid transport across the RPE in the apical to basal direction via intracellular cGMP signaling that likely does not involve PKG.

1354/B569

Ouabain Increases the Transmembrane Resistance of Isolated Sheep Leptomeninges.

A. Filippidis[1], S. Zarogiannis[1], M. Ioannou[2], K. Gourgoulianis[3], P. Molyvdas[1], C. Hatzoglou[1]; [1]Department of Physiology, Medical School, University of Thessaly, Larissa, Greece, [2]Department of Histopathology, University Hospital of Larissa, Larissa, Greece, [3]Department of Respiratory Medicine, University Hospital of Larissa, Larissa, Greece

Objective: Leptomeninges (arachnoid and pia mater) are the upper and lower barriers of subarachnoid space. The existence of ionic permeability is a basic property of epithelia surrounding spaces, which contain biological fluids and are involved in fluid turnover. The aims of this study were to investigate 1) the occurrence of ionic permeability of leptomeningeal tissue in sheep by measuring the transmembrane resistance and thus its potent role in fluid turnover, 2) a possible Na^+ K^+ ATPase activity in leptomeningeal membrane. **Method**: Cranial leptomeningeal tissue was obtained from 26 adult male and female sheeps. Every tissue sample contained one membrane is each side (arachnoid and pia) which together enclosed the subarachnoid space. The leptomeningeal transmembrane resistance (R_{TM}, in $\Omega\bullet cm^2$) was measured with Ussing chambers, under open circuit mode, in controls and after the application of 10^{-3}M ouabain in each side (7 experiments for the arachnoid basolateral side, the side that in vivo faces dura mater, and 6 experiments for the pia basolateral side, the side that in vivo faces brain surface). Histological samples of leptomeningeal tissue were prepared under light microscopy and haematoxilin-eosin staining. **Results**: The mean R_{TM} of the leptomeninges in sheep was found to be 8.85±4.78 $\Omega\bullet cm^2$. The addition of ouabain to the basolateral arachoid and pia mater side, increased the R_{TM} in both sides significantly and rapidly (R_{TM} of 1st minute 15.14±6.12 $\Omega\bullet cm^2$arachnoid and 13.67±2.94 $\Omega\bullet cm^2$pia, $p<0.05$). This effect lasted till the end of the experiment in 30 minutes. The histological preparations revealed homology with the

leptomeninges of humans under light microscopy. **Conclusion**: The leptomeningeal tissue of sheep demonstrates a measurable R_{TM} and thus ionic permeability. The application of ouabain increases the R_{TM} of arachnoid and pia mater. This is indirect evidence for the presence of Na+ K+ ATPase in the arachnoid and pial basolateral surfaces of sheep leptomeninges. Our data suggest, that Na^+ K^+ ATPase channels might be more abundant in arachnoid basolateral membrane.

1355/B570

Basal Constriction during Formation of the Midbrain-Hindbrain Boundary Constriction.

E. Graeden[1,2], J. H. Gutzman[2], H. Sive[1,2]; [1]MIT, Cambridge, MA, [2]Whitehead Institute, Cambridge, MA

Basal constriction is a mechanism of cellular morphogenesis during which the basal surface of an epithelial cell constricts, thus shaping the epithelium. Unlike the more familiar apical constriction, the mechanism of cell shape change via basal constriction during development has not previously been analyzed, although this may be a widely used morphogenetic strategy. In zebrafish, we show that basal constriction occurs during development of one of the earliest brain folds, which separates the midbrain and hindbrain soon after neural tube closure, forming the midbrain-hindbrain boundary (MHB). We have termed the deepest point of this constriction the MHB constriction (MHBC). The MHBC forms concomitant with other aspects of brain morphogenesis, including inflation of the brain ventricles. We show that zebrafish MHBC formation begins as cells at the MHB shorten relative to the surrounding cells. This is followed by basal constriction and apical expansion of a small group of cells that contribute to the MHBC. Brain ventricle inflation is not necessary for basal constriction at the MHBC, demonstrating that this process is actively regulated. Further, basal constriction, but not the earlier cell shortening, requires laminin, as determined by mutant analysis. Preliminary results suggest that basal constriction requires intact microtubules, but not actinomyosin contraction. This study elucidates, for the first time, the molecular regulation of basal constriction necessary for development of the vertebrate brain.

1356/B571

Caspase-8 Regulates Skin Regeneration and Wound Repair.

P. Lee, D. Lee, G. Chen, C. Chan, I. L. Ch'en, C. Jamora; Biology, UCSD, La Jolla, CA

Regulation of epidermal regeneration and homeostasis is achieved by balancing the distinct processes of proliferation vs. differentiation and cell death vs. survival. Disruption of epidermal integrity results in a shift of any one of these balancing acts to initiate a cascade of signals leading to the release of factors that stimulate keratinocyte proliferation, differentiation, survival or death. During homeostatic conditions, we found expression of caspase-8 confined to the granular layer. Upon cutaneous trauma caspase-8 mRNA and protein levels are decreased. In order to investigate the contributions of caspase-8 modulation in response to wounding, we generated mice deficient in caspase-8 in the skin. Analysis of the caspase-8 conditional knockout (cKO) epidermis revealed an increase in the number of proliferating keratinocytes, resulting in a thickened epidermis. Since the cells in the stratum corneum display characteristics of programmed cell death and caspase-8 is known to initiate apoptosis, we hypothesized that caspase-8 abrogation would interfere with the proper formation of the stratified layers of the epidermis. Surprisingly, immunohistochemical analysis revealed that the thickening observed in the caspase-8 cKO epidermis was due to increased proliferation of basal cells as opposed to a blockade in cell death during the terminal differentiation process. Downregulation of caspase-8 in the cKO and wounded skin corresponded to an increase of secreted IL-1α which stimulated epithelial-mesenchymal interactions leading to the enhanced proliferation of basal keratinocytes. Elevated levels of phospho-NFκB were observed by immunoblotting and in the nuclei of suprabasal cells of the caspase-8 cKO skin and quantitative RT-PCR analysis of caspase-8 cKO and wounded skin revealed increased expression of NFκB target and pro-inflammatory genes. Taken together, we have uncovered a molecular mechanism for sensing epidermal perturbations that rely on the modulation of caspase-8 levels thereby initiating an essential epithelial-mesenchymal-leukocytic interaction as a response.

1357/B572

Epidermal Overexpression of Snail Causes Skin Inflammation.

Y. Nakamura, C. Jamora; Division of Biological Sciences, UCSD, La Jolla, CA

Snail is a transcriptional repressor of E-cadherin and inducer of epithelial-mesenchymal transition. Snail plays essential roles in normal development and contributes to diseases such as tumor cell migration and survival. Snail expression is upregulated in some tumor tissues including squamous cell carcinoma (SCC). In SCC, inflammatory phenotypes such as immune-cell infiltration are often observed. Therefore, it is important to elucidate the mechanisms by which inflammatory responses are induced. Given that Snail is upregulated in SCC, it is possible that Snail plays some roles in inflammatory responses. However, little is known about the roles of Snail in inflammatory responses. Here, we found that misexpression of Snail in the epidermis of transgenic mice (K14-Snail Tg) leads to immune-cell infiltration as well as epidermal hyperplasia in skin. The number of various T-cell subclasses is increased both in epidermis and dermis of K14-Snail Tg skin whereas the numbers of macrophages and granulocytes are elevated predominantly in the dermis of K14-Snail Tg skin. In addition, expression of pro-inflammatory cytokines is increased and some inflammatory related signals are activated in skin of K14-Snail Tg mice. These observations suggest that overexpression of Snail in epithelial cells causes inflammatory phenotypes such as immune-cell infiltration, cytokine expression and inflammatory related signal activation. Our results imply that Snail plays important roles in not only cell migration and survival but also inflammation in epithelial tumor tissues such as SCC.

1358/B573

The Human Epidermal Keratinocytes Express, Both In Vivo and In Vitro, the Small Leucine Rich Proteoglycan Decorin.

C. Velez-DelValle, F. Castro-Munozledo, W. Kuri-Harcuch; Dept. of Cell Biology, CINVESTAV-IPN, Mexico City, Mexico

The small leucine-rich proteoglycan decorin, has been described as a major component of mammalian extracellular matrices. Decorin plays an important role in cell differentiation; in various cell types, including cancer cells, this proteoglycan is involved in the regulation of cell attachment, migration and proliferation. When decorin is ectopically expressed, it suppresses the growth of various tumor cell lines in a mechanism that involves the participation of EGF receptors. Since it has been reported that epidermal keratinocytes are strongly influenced

by the action of EGF, and since in the skin, decorin has been located in dermis but not in epidermal keratinocytes we studied whether this cell type expresses this proteoglycan. We show that decorin gene is expressed in cultured human epidermal keratinocytes; its encoded protein was mainly found at the suprabasal layers of human epidermis, suggesting that it might play a role during keratinocyte terminal differentiation. However as shown by real time PCR, the levels of decorin mRNA did not show a significant change between proliferative and confluent cultured cells. Our results also showed that decorin, which is abundant in the extracellular matrices of mesenchymal tissues, was only located in the cytoplasm of differentiating keratinocytes. We also explored the possible interaction between decorin with cytoskeletal proteins, our results show that decorin does not interact with cytokeratins, actin or tubulin.

1359/B574

Apical Constriction and Invagination in the Drosophila Wing Imaginal Epithelium are Induced by Activation of the Canonical Wnt Pathway through Loss of APC2 and APC1.

S. Zimmerman, L. Thorpe, V. Medrano, R. Martz, B. McCartney; Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, PA

Epithelial morphogenesis is driven by coordinated cell shape changes that rely on rearrangements of cytoskeletal architecture. Signal transduction pathways function upstream of morphogenetic events, but the molecular connections between signaling and the cytoskeletal dynamics required for morphogenesis are not well understood. The canonical Wnt signal transduction pathway has well known roles in specifying cell fate during development, but much less is known about its role in morphogenesis. The colon cancer tumor suppressor Adenomatous polyposis coli (APC) is a negative regulator of Wnt signaling and functions in cytoskeletal organization. To determine the cellular consequences of complete loss of APC function in a model epithelium, we generated *APC2 APC1* double null (*APC* null) clones in the *Drosophila* wing imaginal disc. The wing disc is the larval wing primordium, consisting of a single layer of polarized, columnar epithelial cells. Using a combination of genetic interactions and clonal analysis, we show that loss of APC function leads to inappropriate apical constriction, invagination, and outpocketing in the *Drosophila* wing imaginal epithelium due to activation of the Wnt pathway. Although Wnt activation has been reported to upregulate *Drosophila* E-cadherin (*D*E-cadherin) expression in wing imaginal discs, we show that ectopic overexpression of *D*E-cadherin is not sufficient to phenocopy the *APC* null phenotype. Furthermore, we show that apical constriction and invagination of *APC* null tissue are independent of *D*E-cadherin upregulation. We find that phosphorylated myosin regulatory light chain is enriched in the apical cortex of apically constricting *APC* null cells. Because the Rho pathway has been implicated in Myosin II activation, we examined the Rho activator RhoGEF2 in *APC* null tissue and found that it is cortically and cytoplasmically enriched in these apically constricting cells. Our data provide evidence for a novel link between the canonical Wnt pathway and tissue morphogenesis through activation of Myosin II in the wing imaginal disc.

1360/B575

The Influence of Cell Mechanics, Cell-Cell Interactions, and Proliferation on Epithelial Packing.

J. Roeper[1], R. Farhardifar[2], B. Aigouy[1], F. Julicher[2], S. Eaton[1]; [1]Max-Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, [2]Max-Planck Institute for the Physics of Complex Systems, Dresden, Germany

Epithelial junctional networks assume packing geometries characterized by different cell shapes, neighbor number distributions and areas. The development of specific packing geometries is tightly controlled; in the Drosophila wing epithelium, cells convert from an irregular to a hexagonal array shortly before hair formation. Packing geometry is determined by developmental mechanisms that are likely to control the biophysical properties of cells and their interactions. To understand how physical cellular properties combine with proliferation to determine cell packing geometries, we use a vertex model for the epithelial junctional network in which cell packing geometries correspond to stable and stationary network configurations. The model takes into account cell elasticity and junctional forces arising from cortical contractility and adhesion. By numerically simulating proliferation, we generate different network morphologies that depend on physical parameters. Comparing theoretical results to observed cell morphologies in the Drosophila wing disc reveals regions of parameter space for which calculated network morphologies match observed ones. We independently estimate parameter values by quantifying network deformations caused by laser ablation of individual cell boundaries. The vertex model can account qualitatively and quantitatively for the observed packing geometry of the wing disc epithelium and its response to perturbation by laser ablation. Epithelial packing geometry is a consequence of physical cellular properties such as cellular elasticity, cortical contractility and cell-cell interactions, and the disordering influence of proliferation. Currently, we are extending our investigations to the process of hexagonal repacking during pupal development. Cells in the wing become strongly stretched along the proximal-distal body axis before repacking. Performing laser cuts at different time points during development showed that cells are under anisotropic tension during repacking. Thus, anisotropic tension might be the driving force of cell rearrangements in the wing blade.

1362/B577

Analysis of Lectin and Ricin Binding to Human Buccal Cells.

O. A. Vanderpuye, R. Jones, F. B. Ivey; Forensic Science, Albany State University, Albany, GA

Buccal cells lining the oral cavity are research models for oral cancers, drug delivery systems and a DNA source for genetics and forensics. There is limited knowledge of buccal cell membrane protein composition and its roles in susceptibility to infection and interactions with toxins such as ricin. Our objective is to better understand buccal cell polymorphisms and receptors for the toxin ricin and other lectins. Buccal surface glycoproteins were studied by fluorescence microscopy, lectin blots of nitrocellulose transfers of SDS gels and flow cytometry. The Commassie Blue-stained SDS PAGE profile of reduced buccal cell proteins was dominated by a 50-60 kDa doublet absent in non-reduced samples. Approximate molecular masses of the main buccal proteins bound by lectins were Concanavalin A (ConA)(60 kDa), wheat germ agglutinin (WGA) (250 kDa, 70 kDa), peanut agglutinin (PNA) (250 kDa), Ricinus communis agglutinin-I(200 kDa, 80 kDa, 60 kDa), Ricinus communis agglutinin -II (ricin) (200 kDa, 80 kDa), Mackia amurensis agglutinin (MAA) (150 kDa), Sambucus nigra agglutinin (SNA) (200 kDa). Erythrina cristagalli agglutinin(ECA) only weakly stained and Dolichus biflorus (DBA) and soy bean agglutinins (SBA) did not specifically stain buccal proteins. In fluorescence microscopy, buccal cells from different donors bound strongly to

PNA, ConA, and WGA. Lesser binding was observed for SNA, MAA and Tomato lectin (TL)and Jacalin lectin. RCA-I bound buccal cells better than ricin. Ulex Europeus-I (UEA-I), Vicia villosa lectin (VVL), Solanum tuberosum agglutinin (STA), Griffonia simplicifolia lectin II(GS-II) and SBA stained cells from some donors more strongly than others. Datura stramonium agglutinin (DSA), L-PHA and (GNA) bound weakly or not at all and DBA did not bind to buccal cells from tested donors. Flow cytometry showed that WGA and RCA-I bound to all buccal cells but antibodies to the complement receptors, CD59, CD55 and CD46 bound to only 50% of cells. We conclude (a) lectins can be used to study glycoprotein polymorphisms in buccal cells (b) proteins of 200 kDa and 80 kDA are potential major ricin receptors in buccal cells (c) complement receptors vary in expression on buccal cells.

1363/B578

Protease-activated Receptor 2 Induces Ca2+ Entry via Non Capacitaive Calcium Entry in Rat Parotid Glands.

T. Saino[1], E. L. Watson[2], Y. Satoh[1]; [1]Department of Anatomy (Cell Biology), Iwate Medical University, Morioka, Japan, [2]Department of Oral Biology, University of Washington, Seattle, WA

[OBJECTIVE] Protease-activated receptor 2 (PAR2), a G protein-coupled receptor, mediates cellular responses to various proteases in numerous cell types, including salivary glands. However, it is little known that the mechanism of intracellular Ca2+ ([Ca2+]i) dynamics with respect to PAR2 in parotid glands. The issue of whether the stimulation of PAR2 induces responses in parotid glands was examined; with special reference to [Ca2+]i dynamics and nitric oxide (NO) production during PAR2 stimulation. [METHOD] We used adult male rats. Parotid glands were isolated and soaked in Hepes-buffered Ringer's Solution (HR) (pH7.4). Isolated acini keeping essential glandular cell polarity were obtained, and loaded with Indo-1/AM (3 μM) or DAF-2/DA (5 μM) for 45 min at 37°C. Specimens on the cover slip were continuously perfused with HR containing PAR2-activating peptide (AP), SLIGRL-NH2, and/or some specific analogs. We used a real-time confocal microscope (Nikon RCM/Ab). [RESULTS] RT-PCR analysis of mRNA harvested from the rat parotid glands yielded signals for PCR products of the predicted size for PAR-2. PAR2-AP (50 μM) induced an increase of [Ca2+]i. Electron microscopy revealed PAR2-AP-induced exocytosis and fusion of granules (i.e., compound exocytosis). The response to PAR2 activation can be caused by Ca2+ mobilization from intracellular Ca2+ stores. PAR2-AP induced Ca2+ entry was not blocked by gadolinium. The peptide induced Ca2+ release and entry were partially inhibited by a NO synthase inhibitor, L-NAME. However, NO production during the perfusion of HR containing PAR2-AP was not verified in acini. The NO donor, GEA 5024, but not 8-bromo-cGMP, mimicked the effects of PAR2 in activating non capacitative calcium entry (NCCE). [DISCUSSION] These results suggest that parotid glands have PAR2 and the PAR2-induced [Ca2+]I increases regulates via NOS. We proposed that PAR2 activates Ca2+ mobilization and the NCCE pathway.

Cell Polarity I (1364 – 1377)

1364/B580

Symmetry Breaking Polarization Driven by a Cdc42p GEF-PAK Complex.

L. Kozubowski, K. Saito, J. M. Johnson, A. Howell, T. Zyla, D. Lew; Duke University, Durham, NC

In 1952, Alan Turing suggested that spatial patterns could arise from homogeneous starting conditions by feedback amplification of stochastic fluctuations. One example of such self-organization, called symmetry breaking, involves spontaneous cell polarization in the absence of spatial cues. Here we show that the yeast scaffold protein Bem1p promotes symmetry breaking by assembling a complex between a Cdc42p-directed guanine nucleotide exchange factor (GEF) and a Cdc42p effector p21-activated kinase (PAK) that amplifies GTP-loading of the master polarity regulator, Cdc42p. Bem1p has PB1 and SH3 domains necessary for symmetry breaking. Fusion of Bem1p to a GEF bypasses the need for the PB1 domain, while fusion of Bem1p to a PAK bypasses the need for the SH3 domain. We show that both the GEF and the PAK must bind to the same molecule of Bem1p in cis, rather than binding to separate Bem1p molecules in trans, for effective polarization. Although mammalian cells lack a Bem1p ortholog, they contain direct GEF-PAK complexes, and we show that yeast containing an artificial GEF-PAK complex with similar architecture can break symmetry even without Bem1p. How does the GEF-PAK complex contribute to symmetry breaking? Interaction of the complex with GTP-Cdc42p through the PAK would both activate the PAK and recruit the GEF to the immediate vicinity of pre-existing GTP-Cdc42p, leading to auto-amplifying growth of GTP-Cdc42p clusters. Mathematical modeling suggests that such mechanisms can robustly generate a polarized cluster of GTP-Cdc42p from homogeneous starting conditions, as long as there is sufficient GTP hydrolysis by Cdc42p to ensure that any GTP-Cdc42p which diffuses away from the cluster is inactivated. This explains the experimental observation that GTP-locked Cdc42p is unable to promote polarization under physiological conditions. Our findings provide mechanistic insight into an evolutionarily conserved pattern-forming positive feedback pathway.

1365/B581

Dynamic Mechanisms of Cdc42 Recycling Revealed through Live Cell Fluorescence Fluctuation Analysis.

B. D. Slaughter, A. Das, J. Schwartz, B. Rubinstein, R. Li; Stowers Institute for Medical Research, Kansas City, MO

Biological processes such as development, cell motility, and immune response depend on the maintenance of robust cell polarization. Cdc42, a conserved Rho-GTPase, is a master regulator of cell polarization. Cdc42p is recycled by two complementary mechanisms: actin-mediated transport/endocytosis and cytosolic recycling through the Rho-GDP dissociation inhibitor (GDI) (in yeast, Rdi1); however, the molecular interactions controlling these recycling mechanisms are not well understood. Fluorescence correlation spectroscopy (FCS) and cross correlation spectroscopy (FCCS) are powerful techniques to obtain parameters of molecular dynamics, concentration and interactions of mobile proteins in live cells. Here, we implement these techniques, along with fluorescence recovery after photobleaching (FRAP) in living yeast cells to study recycling of Cdc42 at the polarized site and dynamics and interactions of cytosolic Cdc42p. FRAP of GFP-Cdc42p at the polar tip upon Cdc42 mutation, Rdi1 deletion, or LatA treatment to inhibit actin transport reveal that multiple recycling mechanisms work in concert to maintain polarization of Cdc42p. Using FCCS, we observe strong cytosolic interaction of Rdi1p-mCherry and GFP-Cdc42p. While in vitro experiments have shown that Rdi1p forms a stable complex with Cdc42p-GDP, we observe little in vivo interaction of Rdi1p with Cdc42 mutants locked in GDP or GTP forms, suggesting an active GTPase cycle of Cdc42p is necessary for the ability of Rdi1p to extract

Cdc42p from the membrane. FCS results suggest that cytosolic GFP-Cdc42p is present in two dynamic populations. Relative abundance of 'fast' and 'slow' diffusing populations of Cdc42 depends upon the ability of Rdi1p to bind Cdc42 and the GTPase cycle of Cdc42p. In addition, the role of Rdi1p phosphorylation in the regulation of Rdi1p-mediated Cdc42 recycling, and a mathematical model of the spatial relationship of the multiple pathways for Cdc42p polarity maintenance relative to the polar cap will be discussed. In general, challenges of and strategies for fluorescence fluctuation analysis in the popular model system budding yeast will be discussed.

1366/B582

Actin-Mediated Symmetry Breaking Does Not Guarantee Singularity in Budding Yeast.

A. S. Howell, T. Zyla, D. Lew; Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC

Cell polarity is vital for the function of diverse cell types. In many cases it is essential that cells polarize towards one, and only one, site (singularity). Polarization also occurs in the absence of spatial cues, and such "symmetry breaking" is thought to employ positive feedback to amplify stochastic fluctuations in the concentrations of key polarity factors. Studies in the model eukaryote *S. cerevisiae* have suggested two positive feedback mechanisms. The first involves a rapidly-diffusing cytoplasmic complex assembled by the scaffold protein Bem1p, containing a Cdc42p-directed guanine nucleotide exchange factor and a Cdc42p effector p21-activated kinase (PAK). Small clusters of GTP-Cdc42p at the membrane recruit the complex, promoting more Cdc42p GTP-loading in their vicinity (Kozubowski et al. Abstract submitted). The second mechanism involves a slowly-diffusing membrane-bound protein that promotes actin cable nucleation (hereafter called M_{act}). Small clusters of M_{act} promote cable nucleation in their vicinity, and the new cables deliver secretory vesicles containing more M_{act}. To maintain a polarized distribution, M_{act} must be recycled by endocytosis. It is unclear what protein M_{act} represents, or whether this feedback mechanism is sufficient for yeast cell proliferation. To test the potential of the M_{act} model, we constructed an artificial M_{act} by fusing Bem1p (which influences actin cable nucleation through interaction with Cdc42p) to the v-SNARE Snc2p, an integral membrane protein recycled by endocytosis. Bem1p-Snc2p could be polarized by a polarized actin cytoskeleton, but unlike Bem1p it could not be polarized through diffusion. Remarkably, Bem1p-Snc2p promoted polarization and proliferation of yeast in the absence of endogenous Bem1p or spatial cues. Unlike polarization by Bem1p, polarization by Bem1p-Snc2p did not require interaction with PAKs, but was dependent on the formin Bni1p. In addition, Bem1p-Snc2p could polarize to more than one site, sometimes leading to the formation of two buds simultaneously. We conclude that the M_{act} model works but does not guarantee singularity of the polarization process.

1367/B583

Phosphorylation of the Cdc42p-directed GEF Cdc24p Involves Regulated Interaction with a p21-activated Kinase (PAK).

K. Saito, L. Kozubowski, T. Zyla, D. J. Lew; Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC

In the budding yeast *S. cerevisiae*, the guanine-nucleotide exchange factor (GEF) Cdc24p stimulates GTP-loading of Cdc42p to promote polarization and bud formation. Cdc24p undergoes cell-cycle-regulated phosphorylation, catalyzed by p21-activated kinases (PAKs) (Gulli et al., 2000). Cdc24p phosphorylation requires the scaffold protein Bem1p, which directly binds to Cdc24p and PAKs, as well as to many other proteins. We recently found that symmetry-breaking polarization in yeast involves assembly of a Cdc24p-PAK complex (Kozubowski et al., Abstract submitted), suggesting that PAK-mediated Cdc24p phosphorylation may be critical for polarization. We now address the basis for the cell-cycle-regulated nature of that phosphorylation. In principle, changes in Cdc24p phosphorylation could reflect regulation of the relevant kinase and/or phosphatase, or regulation of the associations (Cdc24p-Bem1p or Bem1p-PAK) between members of the complex. To distinguish between these possibilities, we constructed Cdc24p-Bem1p, Bem1p-PAK, and Cdc24p-PAK fusion proteins, which render individual associations irreversible. The fusions were all localized to polarization sites and able to promote symmetry-breaking polarization in the absence of Bem1p. The Cdc24p fusions were phosphorylated in a Cdc42p-dependent manner, and rescued the temperature-sensitivity of *cdc24-1* mutants, indicating that they retain polarity functions. Examination of the fusion protein phosphorylation profiles through the cell cycle suggests that regulated interaction between the PAK and the Bem1p SH3 domain accounts for a majority of the regulation. We are now identifying Cdc24p phosphorylation sites by mass spectrometry and using site-directed mutagenesis to elucidate the functional consequences of that phosphorylation. Gulli et al., 2000, Mol. Cell 6, 1155-1167.

1368/B584

Cdc42 Regulation of Fission Yeast Actin Cytoskeleton.

P. Perez, S. A. Rincón, M. A. Villar, B. Santos; Instituto de Microbiologia Bioquimica, Dpt. Microbiología y Genética, CSIC/Universidad de Salamanca, Salamanca, Spain

Rho GTPases are proteins conserved among the eukaryotic organisms which have a central role in regulating actin cytoskeleton organization. *Schizosaccharomyces pombe* Cdc42 is an essential member of the Rho proteins involved in the regulation of polarized growth and the cell morphology. Although many Cdc42 effector proteins have been described in other organisms, only the p21-activated kinases (PAK), Shk1 and Shk2 have been described in *S. pombe*. We have generated several thermosensitive strains carrying *cdc42* mutant alleles to identify novel proteins regulated by Cdc42. The endogenous $cdc42^+$ gene was replaced by a collection of error-prone PCR amplified *cdc42* ORFs and the temperature-sensitive strains were selected at 37°C. The mutant strain termed *cdc42-879*, contains two point mutations, D76G and L160S. The latter is responsible for the thermosensitive phenotype. *cdc42-879* has depolarized actin patches and weak actin cables at all temperatures. Additionally, Myo52, the *S. pombe* myosin type V, and the formin For3, responsible for the actin cable formation during interphase are mislocalized. *cdc42-879* is suppressed by wild type $cdc42^+$ and by the two genes coding for Cdc42 GEFs: $scd1^+$ and $gef1^+$. On the contrary, none of the *S. pombe* PAK genes suppresses the thermosensitive growth of *cdc42-879* when overexpressed. In a screening for multicopy suppressors of *cdc42-879* thermosensitivity using a genomic library we found $pob1^+$, which encodes the *S. pombe* ortholog of the *S. cerevisiae* Boi proteins. Pob1 is an essential PH-containing protein which localizes to the growing poles and the division site independently of actin. Overexpression of Pob1 restores the viability of in the *cdc42-879* strain at 37°C. Moreover Pob1 also rescues actin cable formation and localization of Myo52, and the formin For3. Cdc42 and Pob1 co-localize and physically interact, and Cdc42 regulates Pob1 stability. Based on these results, we propose that Cdc42 interacts with Pob1 to regulate the actin cytoskeleton.

1369/B585

Conserved NDR Kinase Orb6 Maintains Cell Polarity by Spatially Regulating Small GTPase Cdc42.

M. Das[1], D. Wiley[1], X. Chen[1], K. Shah[2], F. Verde[1]; [1]Mol. Cell. Pharmacology, University of Miami Miller School of Medicine, Miami, FL, [2]Department of Chemistry, Purdue University, West Lafayatte, IN

Conserved NDR kinases have been implicated in the control of cell morphogenesis and cell proliferation in several eukaryotic organisms. The mechanism of morphology control by NDR kinase is, however, still unknown. Using a chemical genetics approach, we show that the fission yeast NDR homolog, Orb6 kinase, maintains polarized cell growth at the cell tips by preventing ectopic activation of the morphology regulator Cdc42 GTPase at the cell sides. Loss of Orb6 kinase activity leads to the rapid recruitment of GFP-tagged Cdc42 GTPase and its effector For3p, normally found only at the cell tips, to the cell sides, to disorganization of the actin cytoskeleton, and to the ectopic activation of cell growth. Consistent with a role for Orb6 kinase in negatively regulating Cdc42 GTPase we observe that intracellular levels of GTP-bound Cdc42 are increased in orb6-25 temperature sensitive mutants. Furthermore, Cdc42 GAP Rga4 over-expression or Cdc42 GEF gef1 deletion, that decrease Cdc42 activity, rescue the morphology phenotype of orb6 mutants. Conversely, increasing intracellular Cdc42 activity by Gef1 overexpression or rga4 deletion is lethal to orb6 mutants but not wild-type cells at semi-permissive temperature. These results are consistent with a model where NDR kinase Orb6 maintains cell polarity by spatially restricting the localization of active Cdc42. Since NDR kinase mutants in higher eukaryotes promotes supernumerary neurite formation and branching, a process dependent on the activity of Cdc42 and RAC GTPases, our observations shed light on a novel, likely conserved mechanism of morphology control.

1370/B586

Tea5p, a Putative PAK-Like Pseudokinase that Localizes to Microtubule Plus Ends and Functions in Cell Polarization.

R. A. Lutz, M. Ellis, F. Chang; Microbiology, Columbia University, New York, NY

Cell polarization is crucial for many biological processes like development, cell growth, and cell migration. In the fission yeast *Schizosaccharomyces pombe*, the rod-shaped cells begin polarized growth at the old end after division, and then initiate a second site of growth at the new end in G2-phase, in a process called new end take off (NETO). Here in a genetic screen for novel NETO mutants, we identify a new factor tea5p, which has putative Cdc42/Rac interactive binding (CRIB) regions and is related to the PAK/STE20 protein kinase family. It is likely that tea5p is a "pseudokinase", as it lacks key residues which are predicted to be required for kinase activity. Tea5p shows a dynamic localization pattern: small spots are transported at the plus ends of microtubules in a kinesin-dependent manner and are then deposited at the cell cortex, very much like other NETO factors tea1p and tea4p. Cells lacking tea5p have defects in localizing active cdc42p GTPase and the formin for3p to the second, non-growing end and in the cortical distributions of tea1p and tea4p. Interphase microtubules in *tea5* mutants appear normal. One current model is that tea5p works together with cdc42p, tea1p and tea4p to modulate phosphorylation events for the initiation of cell polarization.

1371/B587

Aging in Fission Yeast: Cell Polarity and Segregation of Damaged Proteins.

N. Erjavec, F. Chang; Microbiology, Columbia University, New York, NY

One of the challenges faced by proliferating cells is represented by the correct distribution of damaged, and potentially noxious, substrates at the time of division. In budding yeast, the asymmetric segregation of oxidatively damaged proteins leads to a differential fitness and life span of the offspring. Although fission yeast S. pombe appear to divide more symmetrically, in these cells, siblings also exhibit asymmetric replicative lifespan potential. Staining for carbonylated proteins shows that oxidatively damaged proteins are preferentially localized to the cell poles and division plane in a cell cycle-dependent manner and are asymmetrically segregated upon cytokinesis. A candidate screen revealed the involvement of microtubules and polarity factors responsible for NETO (New End Take Off) in this polarized distribution of oxidized proteins. For instance, these are diffusely localized in tea1 or tea2 mutants. Interestingly, the relocalization is dependent also on the Hsp104 chaperone as well as on a functional proteasome. The effect of these factors on lifespan will be examined. These results suggest that asymmetric segregation of oxidized proteins is coordinated by the cytoskeleton, components of the polarity machinery, adaptors (Hsp104) and the degradatory machinery (proteasomes).

1372/B588

Anterior Embryonic Polarity Is Maintained by Dynamin.

Y. Nakayama[2], J. Shivas[1], D. S. Poole[1], J. Kulkoski[1], J. Squirrell[3], J. Schleede[1], A. R. Skop[1]; [1]Genetics, University of Wisconsin-Madison, Madison, WI, [2]Molecular Cell Biology, Chiba, Japan, [3]Laboratory for Optical and Computational Instrumentation, University of Wisconsin-Madison, Madison, WI

The PAR polarity factors and the actin cytoskeleton are crucial for generating cell diversity throughout development (1,2). Recent evidence from a genome-wide RNAi screen in C. elegans reveals that anterior polarity cues (PAR-6, PKC-3, PAR-3, and CDC-42) can also specifically regulate the endocytic machinery (3). Current models for the role of PAR proteins in polarity suggest that PAR-dependent membrane recycling is required to generate or possibly maintain a plasma membrane domain and boundary (4), yet the links between the PAR proteins, endocytic machinery and the actin cytoskeleton during development have not been identified. Dynamin is a large GTPase that plays key roles in both endocytosis and actin dynamics and therefore a possible factor that could connect these events to cell polarity pathways. Here, we show that C. elegans dynamin, DYN-1, is required to maintain the anterior polarity factors PAR-6, RHO-1 and CDC-42 as well as the spindle pole movements in the developing embryo. Interestingly, DYN-1-GFP foci are enriched in the anterior cortex of the embryo where endocytosis primarily occurs. Lastly, DYN-1 regulates anterior membrane and actin comet dynamics specifically during the polarity maintenance phase. Our results demonstrate a dynamin-dependent mechanism for the spatial and temporal regulation of polarity, endocytosis and actin dynamics in the anterior of the embryo, contributing to the precise localization and maintenance of polarity factors within a dynamic plasma membrane. References: 1. Betschinger, J. & Knoblich, J.A. Dare to be different: asymmetric cell division in Drosophila, C. elegans and vertebrates. Curr Biol 14, R674-685 (2004). 2. Munro, E.M. PAR proteins and the cytoskeleton: a marriage of equals. Current opinion in cell biology 18, 86-94 (2006). 3. Balklava, Z., Pant, S., Fares, H. & Grant, B.D. Genome-wide analysis identifies a

general requirement for polarity proteins in endocytic traffic. Nature cell biology 9, 1066-1073 (2007). 4. Wissler, F. & Labouesse, M. PARtners for endocytosis. Nature cell biology 9, 1027-1029 (2007).

1373/B589

Mechanics of Cellular Polarization in the C.elegans zygote.

M. Mayer[1, 2], S. Grill[1, 2]; [1]MPI of Molecular Cell Biology and Genetics, Dresden, Germany, [2]MPI of the Physics of Complex Systems, Dresden, Germany

Asymmetric cell divisions are a fundamental prerequisite for generating cellular diversity in developing organisms. During the first division of the C. elegans zygote, the symmetry-breaking event that dictates polarity is thought to be an anterior directed cortical flow resulting from a contractile and dynamic cortical actomyosin meshwork. This flow serves to segregate both cytoplasmic and cortical cell fate determinants between the emerging anterior and posterior domains, in particular establishing an asymmetry in PAR protein distribution, which is maintained and signaled to downstream processes such as spindle positioning and asymmetric cytokinesis. Although a local weakening of the posterior cortex appears to be the trigger that initiates cortical flows, the physical basis of and regulatory mechanisms behind these flows remain poorly understood. We examined the nature of cortical tension in the single cell stage C. elegans actomyosin meshwork and its relation to cortical flows. To reveal the tension governing the cortex in a particular direction, we severed the actomyosin meshwork along a line perpendicular to this direction using pulsed UV laser ablation. Analyzing the kinetics of the elastic response of the cortex subsequent to the cut allows us to quantify both local tension and local stiffness. We find that tension is isotropic prior to symmetry breaking, but relaxes along the anterior-posterior axis in response to symmetry breaking during the period of cortical flows. Because tension remains high in the direction orthogonal to the AP axis, our work indicates that a weaker posterior cortex serves to reduce tension in one direction, but not the other. This reduction in turn generates the tension gradient that is responsible for driving cortical flows. Finally, we use our assay to show that the amount of cortical tension is regulated by the RhoGTPase cycle, probing RNAi conditions of both a GAP and a GEF. In summary, our work suggests a mechanism by which local regulation of tension and contractility is translated into cortical flows that establish anterior-posterior polarity preceding the first asymmetric cell division.

1374/B590

PSL-1 is a Regulator of PAR-polarity in the C. elegans embryo.

C. Hoege, A. T. Constantinescu, A. A. Hyman; MPI-CBG, Dresden, Germany

Cells in an embryo get polarized at distinct points of development to differentiate into various cell types of a multicellular organism. In C. elegans embryos, polarity is established at the one cell stage and involves the asymmetric rearrangement of the acto-myosin network of the cell cortex. In response to this asymmetry PAR proteins are differentially recruited to the cell cortex and this leads to the formation of two distinct cortical domains which signal an asymmetric cell division and the segregation of cell fate factors. Although the localization of PAR proteins is well described in many cell types, little is known how these domains are maintained and how they signal downstream. In an effort to identify regulators of PAR polarity we screened for synthetic lethal components of temperature sensitive PAR mutants and identified PSL-1. Transgenic expression of PSL-1 showed that it is a novel component of the posterior cortex with PAR like localization dynamics. Interestingly, biochemical experiments showed that it associates with PAR-6 and PKC-3. PSL-1 mutants are hypersensitive to PAR protein level changes and this indicated that PSL-1 is a regulator of PAR polarity and protector of the germ line lineage.

1375/B591

EXC-5 Maintains the Apical Surface of the C. elegans Excretory Canal Tubules.

B. Mattingly, M. Buechner; Dept. of Molecular Biosciences, University of Kansas, Lawrence, KS

Nematode excretory canals are thin hollow tubules that run the length of the animal, and are built from a single cell. Extension and maintenance of these tubules requires concomitant growth of the basal outer surface of the canals and of the apical luminal surface. In *C. elegans*, loss-of-function (*lof*) mutations in the *exc-5* gene cause the canal lumen to swell into large fluid-filled cysts predominantly at the tips of the extended tubules. These cysts form as a result of a mechanical failure of the actin-based cytoskeleton lining the canal apical surface. Overexpression of EXC-5 causes a gain-of-function (*gof*) phenotype in which the apical surface is intact, but the basal surface shows defects reminiscent of mosaic mutants in the laminin alpha chain. The canal basal surfaces cannot extend, while the lumenal surface has a normal diameter and is wrapped inside the excretory cell body to form a convoluted tubule phenotype.</p> EXC-5 is a putative guanine nucleotide exchange factor of the FGD protein family. Defects in FGD1 and FGD4 cause the human genetic disorders FacioGenital Dysplasia and Charcot-Marie-Tooth disease, respectively. Genetic interactions suggest that EXC-5 transmits a signal from the EXC-9 LIM-domain protein to the MIG-2 GTPase to regulate placement of the apical cytoskeleton.</p> Through a genetic suppression screen, we have identified and are mapping two new suppressors of *exc-5* that attenuate the EXC-5 *gof* signal to allow normal growth of the excretory canals in the presence of excess EXC-5 function.</p> In order to identify subcellular processes altered by *exc-5 gof* and *lof* mutations, we have created nematodes expressing subcellular markers in the excretory canals. Labeled proteins include EEA-1, CHC-1, CDC-42, RAB-5, RAB-7, RAB-11, GRIP, and GLO-1 (thanks to Barth Grant for constructs). We have observed the location and organization of subcellular compartments (early endosomes, endosomes, lysosomes, apical surface) along the length of the excretory canals, including general cytoplasmic as well as punctate expression. We are characterizing the placement of these proteins in wild-type and mutant canals.

1376/B592

Establishment of Cell Polarity via Cleavage Divisions in Sea Urchin Embryos.

L. Alford, L. H. Henson, H. F. McKay, D. Burgess; Biology, Boston College, Chestnut Hill, MA

In protostomes, cell polarity is present at the first cell division or after fertilization whereas deuterostome eggs are thought to show minimal cortical polarity prior to cell junction formation at the 16-cell stage. We now show establishment of cell polarity as early as the first cleavage division in deuterostome sea urchin embryos, when new plasma membrane is inserted into the furrow. We find, using the apical markers Gm1, integrins, and the aPKC-Par6 complex, that cells are polarized upon insertion of distinct basolateral membrane at the first division.

Live cell imaging of Gm1 shows that the new basolateral membrane lacks Gm1 and this polarized localization is independent of cell-cell contact and the actin cytoskeleton. Similarly, Par6 and CDC42 are found exclusively on the outer, microvillus covered apical surfaces, and their localization is dependent on calcium-dependent cell-cell adhesion, actin, and aPKC activity. Interestingly, Gm1 localization is independent of aPKC activity but is sensitive to myosin filament assembly. This early morphological polarity reflects precocious functional polarity. We have found that isolated cleavage blastomeres exhibit polarized fluid phase endocytosis only on the Gm1, integrin, microvillus-containing apical surface. A role for the PAR complex in cleavage plane determination was shown by interfering with aPKC activity, which results in spindle defects including eccentric spindles, multipolar spindles, and loss of normal blastomere organization. These studies suggest that cell polarity is established at the first cleavage in a model deuterostome and is achieved by directed insertion of basolateral membrane in the cleavage furrow. Maintenance of this cell polarity by actin, cell adhesion, the PAR complex, and aPKC activity is required for proper cytokinesis and early development.

1377/B593

Asymmetric Fission of Giant Lipid Vesicles to Produce Non-identical Daughter Vesicles.

M. Andes, C. D. Keating; Chemistry, the Pennsylvania State University, University Park, PA

The long-term goal of this research is to elucidate possible consequences of cell differentiation by developing an experimental model system capable of generating and maintaining (bio) chemical polarity. Polarity, the defining of the different ends of the cell, is an essential step for cellular processes such as growth, division, differentiation, and migration. In this work, polyethylene glycol (PEG)/dextran aqueous two-phase systems (ATPS) are encapsulated in giant vesicles (GVs) to generate a cytoplasm mimic. The ATPS mimics the macromolecular crowding and chemical heterogeneity of biological cytoplasm. Biomolecules such as proteins or DNA can be localized to sub-regions of the vesicle interior by partitioning between the aqueous phases, and the degree of localization is controlled by slight adjustments in temperature, osmotic pressure, or pH. Additionally, we have observed shape transitions in response to external stimuli. For instance, by increasing the osmolarity and temperature of the system we are able to generate asymmetrically "budded" vesicles resembling pre-divisional bacterial cells. This method of symmetry-breaking of the aqueous interior has been utilized to generate asymmetry in both the aqueous interior and the lipid membrane surrounding the vesicle. Exposure of ATPS-containing GVs to extremely hypertonic conditions results in fission of the budded vesicles to generate two non-identical daughter vesicles that are different in both membrane and interior composition, which will be discussed here.

Germ Cells & Fertilization (1378 – 1399)

1378/B594

Comparison of Ovarian Luteinizing Hormone Receptors among Mouse Strains.

O. Suzuki, M. Koura, Y. Noguchi, K. Uchio-Yamada, J. Matsuda; Laboratory of Experimental Animal Models, National Institute of Biomedical Innovation, Ibaraki, Japan

Induction of superovulation by gonadotropin treatment is widely used in reproductive experiments. However, our previous study (Reprod. Fertil. Dev., 8:975-80, 1996) demonstrated that the differential response among various mouse strains to gonadotropins still poses a major problem for practical applications. To elucidate the mechanism underlying the strain difference in superovulation induction, we planned qualitative and quantitative comparisons of the gonadotropin receptors in various mouse strains. We have already reported that no difference was found in the FSH-receptor amino acid sequences and their expression among mouse strains (AALAS meeting, 2008). In this study, we performed a quantitative comparison of ovarian LH-receptor mRNA among mouse strains. First, we confirmed the nucleotide sequences of LH-receptors using RT-PCR with testis RNA from 12 strains of mice in combination with direct sequencing of the PCR products. The LH-receptor cDNA sequences in all of the strains examined were essentially the same as that of C57BL/6J (accession number: NM_013582), except that the 491st nucleotide was C in C57BL/6J, DBA/1, DBA/2, SJL, NZB, and NZW and T in BALB/c, C3H/HeJ, 129x1/Sv, CBA/N, AKR/N, and A/J. Total RNA was extracted from the ovaries of 4-week-old females (five mice per strain) belonging to six strains (C57BL/6Cr, A/J, DBA/1, DBA/2, C3H/HeJ, and BDF1) 48 h after PMSG treatment. The amount of LH-receptor mRNA was quantified using quantitative real-time RT-PCR, with normalization by the amount of GAPDH mRNA. The amounts of transcripts did not differ significantly among strains judged using one-way ANOVA (p=0.053). Therefore, the strain difference in the response to gonadotropin treatment is not likely due to the differential abundance of ovarian LH-receptors. Now, we are attempting to compare protein abundance among mouse strains using Western blot analysis. This work was supported by a grant from the Ministry of Health, Labour and Welfare of Japan.

1380/B596

Cortactin, a Functional Partner of Arp2/3 Complex, is Required for the Completion of the Mouse Egg Activation.

D. Yu[1,2,3], H. Zhang[1,2,3], S. Zhan[1,2,3]; [1]Marlene Stewart Greenebaum Cancer Center, University of Maryland at Baltimore, School of Medicine, Baltimore, MD, [2]Center for Vascular and Inflammatory Diseases, University of Maryland at Baltimore, School of Medicine, Baltimore, MD, [3]Department of Pathology, University of Maryland at Baltimore, School of Medicine, Baltimore, MD

The actin cytoskeleton plays a fundamental role in embryonic development, including cortical changes associated with maturation of gametes and egg activation upon fertilization. However, the mechanism regulating the actin assembly in the early embryonic development is still poorly understood. Heterozygous mice bearing a disrupted allele of cortactin, an Arp2/3 complex activator that promotes the formation of branched actin filaments in the cell cortex, failed to show any apparent phenotype, although embryonic fibroblasts derived from heterozygous mice showed decreased membrane ruffle formation in vitro. Yet, neither homozygous postnatal animals nor two-cell embryos carrying a homozygous genotype could be obtained. The failure to obtain homozygous embryos was not due to possible defect in sperm or egg maturation as the gametes carrying the mutated cortactin allele were effectively transmitted into the offspring. Analysis of single-cell eggs revealed a gradual decrease in homozygous zygotes upon fertilization, with 20% at 13 hours and only 1% at 22 hours post human chorionic

gonadotropin (hCG) injection. Furthermore, expression of cortactin along with Arp3, a subunit of Arp2/3 complex, was dramatically increased at 22 hours post hCG. The fertilized eggs with low cortactin expression often displayed abnormality in morphology. In normal eggs cortactin was mainly distributed in the area around the chromosome and the polar body. Our data suggest that cortactin play a distinct role in mammalian egg activation.

1381/B597

Differentiation of Nurse Cells and Oocytes in the Polychaete Worm, Ophryotrocha Labronica: On the Evolutionary Conservation of Oogenic Cysts in Animals.

J. L. Brubacher[1,2], E. Huebner[2]; [1]Faculty of Humanities and Sciences, Canadian Mennonite University, Winnipeg, MB, Canada, [2]Department of Biological Sciences, University of Manitoba, Winnipeg, MB, Canada

Polarized oogenic cysts are clonal syncytia of germ cells in which a subset of sister cells (cystocytes) differentiate not as oocytes, but instead as nurse cells: highly polyploid cells that support oocyte growth and development. The intricate machinery required to establish and maintain divergent cell fates within a syncytium, and the importance of associated oocyte patterning for subsequent embryonic development, have made polarized cysts valuable subjects of study in developmental and cell biology. Nurse cell/oocyte specification is best understood in insects, particularly *Drosophila melanogaster*. However, polarized cysts have evolved independently in several other animal taxa, including the annelid worms. Our research focuses on the differentiation of female cystocytes in one such worm, the polychaete *Ophryotrocha labronica*. These worms are remarkable for their elegantly simple 2-cell cysts, composed of a single oocyte and nurse cell, making them a fascinating alternative to insects as subjects of study. We have constructed digital 3D models from electron micrographs of serially sectioned ovarian tissue, to illustrate the process of cystocyte differentiation in *O. labronica*. These models show that 2-cell cysts arise by fragmentation of larger "parental" cysts, rather than as independent units. Various characteristics of the parental cysts show that, whatever similarities may exist between the molecular determinants of cystocyte fate in *O. labronica* and insects, the cytological contexts in which these determinants are deployed are very different. In light of these differences, and the plasticity of cyst structure in other animal phyla, we suggest that it is time to re-evaluate the views that cyst structure is well-conserved, and that the cyst stage itself is inherently necessary for animal oogenesis.

1382/B598

A Two Stage Dependence on 1-Methyladenine for Maturation of Starfish Oocytes.

M. Terasaki; Cell Biology, Univ Connecticut HC, Farmington, CT

In starfish, oocyte maturation is caused by the hormone 1-methyladenine (1-MA). We found an unexpected 1-MA dose dependency of isolated oocytes of the starfish Asterina miniata. 0.5 μM 1-MA or greater causes 100% GVBD. GVBD usually occurs at 20-30 minutes; to simplify discussion, we will use 24 min. If two groups of oocytes are put into 0.005 μM and 0.5 μM at the same time, then the first group switched to 0.5 μM at 10 minutes (or any time before), both groups will undergo GVBD at 24 min. This indicates that a very low concentration of 1-MA can start the maturation process, while a higher concentration is required to finish it. 1-MA binds to a Gαi protein coupled receptor on the oocyte surface. One possible mechanism for this two stage dependence on 1-MA is that αi subunits and βγ subunits freed by the 1-MA receptor activate two separate downstream pathways but that βγ subunits are more potent in activating their downstream targets than αi subunits are. In support of this idea, we found that injection of βγ subunits speed up GVBD timing in response to 0.5 μM 1-MA. As for biological function, this dose dependency may help to synchronize maturation throughout the ovary despite large gradients of the neuropeptide GSS.

1383/B599

Formation and Function of Intercellular Ring Canals in Drosophila.

A. M. Hudson[1], S. Airolodi[1], L. Cooley[1,2,3]; [1]Genetics, Yale University, New Haven, CT, [2]Cell Biology, Yale University, New Haven, CT, [3]MCDB, Yale University, New Haven, CT

Animal gametes develop in syncycial clusters of cells connected by stable intercellular bridges that form on arrested mitotic cleavage furrows. Similar intercellular bridges are present between somatic epithelial cells in *Drosophila*. We are characterizing the components of intercellular bridges in both *Drosophila* germline and somatic cells, and the movement of cytoplasmic components between cells. *Drosophila* female germline intercellular bridges, called ring canals, have a rim of highly dynamic F-actin. During development, the ring canal lumen expands from 1 to 10 microns, in a process requiring Arp2/3-dependent actin polymerization. Mutations in the kelch gene result in an overabundance of disorganized F-actin that nearly fills the lumen of the ring canal. The *Drosophila* Kelch protein (the founding member of the BTB-Kelch family) colocalizes with and crosslinks ring canal F-actin. Here we show Kelch also associates with Cullin3 (similar to other BTB-Kelch proteins), and likely functions as a substrate adaptor in a Cullin Ring Ligase (CRL). Loss of Cul3 in the germline resulted in ring canals with excess F-actin, and a prominent redistribution of Kelch protein into the ring canal lumen. Kelch protein was also present at abnormally high levels in *Cul3* mutants, consistent with it being a substrate-binding component of a CRL. YFP-Cullin3 mirrors Kelch ring canal localization both spatially and temporally. These results suggest that targets of the Kelch/Cul3 CRL are targeted for proteasome-mediated degradation in order to allow normal actin depolymerization in ring canals. Ring canals 0.3 microns in diameter are present in ovarian somatic follicle cells and in imaginal discs. Previous reports suggested that Pavarotti-KLP (Pav-KLP) persists in follicle cell ring canals. In addition, a GFP-protein trap in Visgun (a predicted transmembrane glycoprotein) revealed localization to ring canal-like puncta in post-mitotic follicle cells. We have confirmed the localization of both Pav-KLP and Visgun to follicle cell ring canals using immunoEM. Interestingly, follicle cell ring canals are often present at the junction of three cells, suggesting a novel mechanism for intercellular communication in epithelial cells.

1384/B600

In-Silico Drosophila Melanogaster Early Patterning and Morphogenesis.

Y. Jamali, M. Mofrad; Bioengineering, University of California Berkeley, Berkeley, CA

There is few knowledge about how cellular processes integrate with one another and with patterning information in the embryo to create complex biological structures from simple sheets of cells. One of the most widely used model organisms in developmental biology research is the Drosophila melanogaster, or fruit fly. We know that localized changes in mechanical properties or generation and transmission of mechanical forces can drive large scale reshaping of tissues. There are some studies on the mechanical properties of developing organisms, but, none of these have looked into Drosophila oogenesis. We developed a computational model to study the minimum biomechanical conditions that can create this global morphological change. We try to reconstruct and simulate the morphological processes experienced in the drosophila oogenesis during stages 4-12a with a simple biomechanical model. This investigation focused on two important morphological changes, namely elongation of egg chamber along the A-P axes in the stages 4-7 and reorganization of follicle cell epithelium in stage 8-12a. The result shows that for morphological rearrangement we do not need a global asymmetry and some change in the local cells may be sufficient to create such global rearrangement. In other words, only a local communication between immediate neighbor cells can create this rearrangement, whereas for the elongation process a more global communication is required among the cells.

1385/B601

Programmed Cell Death in Drosophila melanogaster Germ Cells.

L. D. Grantham[1], Y. Yamada[2], C. R. Coffman[1]; [1]Genetics Development and Cell Biology, Iowa State University, Ames, IA, [2]Janelia Farm Research Campus, Howard Hughes Medical Institute, Ashburn, VA

Programmed cell death (PCD) is essential for the proper development of multicellular organisms. *Drosophila melanogaster* germ cell development can be used as a model to study the mechanisms that regulate PCD. During embryogenesis, germ cells migrate from the posterior midgut to the developing gonad. While the germ cells are migrating, approximately half of them die by PCD. To determine when during embryogenesis PCD takes place, germ cells were labeled using an anti-Vasa antibody and counted using light microscopy. Staging of embryos was done based on morphological criteria. PCD of germ cells takes place during stages 10-12 of embryogenesis, with the number of germ cells being reduced from an average of 34 germ cells per embryo to an average of 17 germ cells per embryo. Live imaging using hyperpectral confocal microsopy of embryos with vasa-eGFP labeled germ cells allows for a dynamic look at germ cell migration and death.

1386/B602

A Subset of Genes is Upregulated during Zygote Genesis in S. cerevisiae.

P. Jaiswal[1], D. Wu[2], A. Tartakoff[1]; [1]Department of Pathology and Cell Biology Program, Case Western Reserve University, Cleveland, OH, [2]Monash Institute of Medical Research, Monash University, Melbourne, VIC, Australia

The formation of yeast zygotes provides a model for investigation of aspects of cell fusion and fertilization in other organisms. In this process, the two parental populations of organelles redistribute, in most cases by fusing cis-to-trans. To identify genes which are required, we have crossed wildtype strains in complete synthetic medium. RNA was harvested at successive time points (0-3.5 hr) and used to generate probes for interrogation of Affymetrix microarrays. Changes due to stimulation with mating factor were eliminated by following, in parallel, cultures of cells in which the two mating types were separated by a Millipore filter. A subset of 23 genes shows specific upregulation and the kinetics of their upregulation allow them to be assigned to 2-3 subgroups. The corresponding genes are not required for mitotic growth and most of them do not depend on Kar4p or Ste12p.

1387/B603

A Novel Eif4e-Binding Protein Is Required for Spindle Positioning during C. Elegans Embryogenesis.

W. Li, L. R. Debella, L. S. Rose; Molecular and Cellular Biology, University of California Davis, Davis, CA

Translational control of messenger RNA allows the immediate and direct adaptation of protein levels and plays a critical role in developmental events that take place largely in the absence of transcription. Several mechanisms for translational regulation have been described. In the cap-dependent mode, the 5' cap binding protein eIF4E functions in conjunction with eIF4G to mediate the recruitment of the 40S ribosomal subunit. Several eIF4E binding proteins (4E-BPs) inhibit translation initiation by competing with eIF4G for binding to eIF4E, in a reversible and highly regulated manner.   Here we show that a novel eIF4E-BP, SPN-2, is required for early embryonic divisions in *C. elegans*, which occur in the absence of transcription and depend upon maternally deposited mRNAs and proteins. The *spn-2* mutation was first identified in a screen for maternal effect lethal mutations that result in defects in asymmetric division. Some one-cell mutant embryos exhibit spindles perpendicular to the polarity axis. Those that divide normally at first cleavage show spindle orientation defects at the two-cell stage. In addition *spn-2* mutant embryos show defects in other cytoskeleton-dependent processes such as membrane furrowing, interphase nuclear positioning and centrosome attachment. Molecular cloning showed the *spn-2* gene encodes a protein with limited homology to eIF4E-BPs, and biochemical assays indicate that SPN-2 is a bona fide eIF4E-BP. Consistent with a role in translation, SPN-2 localizes to the cytoplasm and to P granules, ribonucleoprotein particles that localize to germ-line precursor cells during asymmetric division. We also found that MEI-1, a microtubule severing protein, is ectopically localized to spindles in *spn-2* embryos. Others have shown that MEI-1 protein is normally degraded by a ubiquitin-dependent pathway at the end of meiosis, and that ectopic expression in mitosis results in spindle positioning defects. We find that depletion of MEI-1 in the *spn-2* background rescues some defects. We are currently testing whether SPN-2 regulates MEI-1 expression via its degradation pathway or more directly regulates *mei-1* mRNA via a role in translation control.

1388/B604

Sequential Redistribution of Organelles during Zygote Genesis in S. cerevisiae.

P. Jaiswal, M. Khan, A. Tartakoff; Department of Pathology and Cell Biology Program, Case Western Reserve University, Cleveland, OH

Zygote formation in yeast involves cell wall and plasma membrane dissolution, followed by redistribution of parental organelles. To investigate the order and mechanisms which accomplish organelle redistribution, we have studied crosses of strains in which individual marker proteins are tagged with GFP vs RFP. Samples were either fixed at regular intervals for quantitation or were observed by real-time imaging. These protocols show that soluble proteins equilibrate before ribosomes, which begin to equilibrate when nuclei establish contact.

Mitochondria equilibrate later, followed by peroxisomes. Minute-by-minute imaging show that mitochondria fuse with each other, with both outer and inner membranes fusing simultaneously. Their fusion appears to be delayed by a medial partition which includes septins.

1389/B605

Null Mutation of the Transcription Factor Inhibitor of DNA Binding 3 (Id3) Causes Male Infertility and Selectively Affects Gene Expression in the Mouse Epididymis.

M. Carroll, B. Robaire; Pharmacology and Therapeutics, McGill University, Montreal, QC, Canada

The epididymis, a component of the excurrent male reproductive tract, is a highly specialized tissue that functions in the transport, maturation, protection, and storage of spermatozoa. The functional segmentation of the epididymis into 4 distinct regions is reflected at the molecular level by complex region-specific gene expression profiles; these are responsible for establishing the microenvironment required to carry out sperm-related functions. Id3 is a transcription factor that acts as a dominant-negative regulator of other transcription factors by sequestering them, thus rendering them unable to bind DNA. Previous work done in our laboratory suggests that Id3 may act as an important regulator of many downstream genes associated with the region-specific differences that are the hallmark of the epididymis. The objective of these studies was to determine the effect of Id3-deficiency on reproductive outcome and epididymal gene expression profiles. Mating studies were done to examine reproductive outcome. In contrast to matings of wild-type animals, crossing Id3-null males with wild-type females leads to serious adverse reproductive effects including decreased fetal weights and litter sizes, increased rates of early and late fetal demise, as well as developmental delays and malformations. Importantly, live litters are not produced from these crosses. We employed microarray technology to assess genome-wide expression profiling of mRNAs expressed in the mouse caput and cauda epididymidis from both Id3+/+ and Id3-/- mice. Principal component analysis (PCA) indicates that gene expression signatures for these 2 genotypes are distinct from each other in each epididymal region, occupying very different spaces within the PCA plot. However, the predominant effect of the Id3 null mutation is in the cauda region where the expression of many transcription factors is markedly affected. These results provide novel insight into the role of Id3 in the mouse, suggesting that Id3 may coordinate gene expression programs in the male reproductive system associated with the generation of healthy offspring. Supported by CIHR.

1390/B606

Characterization of New Genes Required for Sperm Tail Formation in Drosophila.

J. E. Rollins[1,2], L. Fabian[2], J. Brill[2,3], B. Wakimoto[4]; [1]Division of Science, College of Mount Saint Vincent, Riverdale, NY, [2]Program in Developmental & Stem Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada, [3]Department of, University of Toronto, Toronto, ON, Canada, [4]Department of Zoology, University of Washington, Seattle, WA

Drosophila sperm tail formation required the highly polarized addition of membranes and growth of microtubules in the elongating axoneme. We previously showed that the membrane lipid phosphatidylinositol 4,5-bisphosphate is required for sperm tail formation and axoneme assembly (Wei et al., 2008). Moreover, membrane trafficking is required for formation of elongated sperm (Farkas et al., 2003 Xu et al., 2002). To identify new genes involved in sperm tail formation, we screened candidate genes previously identified from a collection of viable EMS mutants (Wakimoto et al., 2004). Of 20 mutants screened, 8 gave strong reproducible phenotypes indicating a defect in sperm tail formation. Immunofluorescence revealed a lack of polarity of the developing spermatid cysts as well as defects in spermatid elongation, nuclear shaping and actin organization. The novel collection of genes will provide insight into molecular mechanisms involved in male germ development, cell polarity and coordination of membrane and cytoskeletal rearrangement during development.

1391/B607

Nucleoside Transporter Slc29a1 Is Not Required for the Accelerating Action of Adenosine Analogs on Sperm Motility.

L. Burnett[1], A. Moss[2], J. Unadkat[2], D. Babcock[1]; [1]Physiology and Biophysics, University of Washington, Seattle, WA, [2]Pharmaceutics, University of Washington, Seattle, WA

Adenosine in female reproductive fluid is a candidate modulator of the motility of spermatozoa as they progress toward the site of fertilization. Past work demonstrated that the adenosine analog 2-chloro-deoxyadenosine (Cl-dAdo) accelerates the flagellar beat of mouse sperm by a cAMP/PKA-mediated pathway with a pharmacological sensitivity profile that suggested possible involvement of a Slc29a (ENT) nucleoside transporter. Using qRT-PCR we now demonstrate predominant expression of Slc29a1 in adult mouse testis; Slc29a1 has 160- and 32-fold greater expression than the other surface-membrane ENT transporters Slc29a4 and Slc29a2. Although Slc29a1 mRNA is expressed in the testis, Slc29a1 protein was not found on mature sperm using immunocytochemistry. Moreover, wildtype and Slc29a1-null mice accelerate at similar rates (1.5-2.0 Hz min-1) in response to Cl-dAdo indicating that Slc29a1 is not required for responses to Cl-Ado. Consistent with this observation, the accelerating action of Cl-dAdo resists the Slc29a-selective inhibitor nitrobenzylthioinosine (NBTI; 10 μM). The accelerating action of Cl-dAdo additionally resists replacement of external Na+ with NMDG indicating that Slc28a concentrative nucleoside transporters (CNTs) probably also are not required. Interestingly, the Adora3-selective agonist IB-MECA (100 μM) is nearly as effective as Cl-dAdo in accelerating sperm beat frequency, suggesting a possible role for cell surface Adora3 adenosine receptors in Cl-dAdo-mediated responses in sperm motility. Support from U54-HD12629 of the SCCPRR program of NICHD. L.A.B. supported in part by 5-T32-HD007453.

1392/B608

Rho Kinase (ROCK) and G α12/13 Function as Key Proteins in Crosstalk between Integrin- and G Protein Coupled Receptor-Dependent Signaling Events in Ascidian Sperm Activation.

J. Corleto, R. Koch; Biological Sciences, California State University, Fullerton, Fullerton, CA

During fertilization, sperm cells are activated by binding to egg follicle cells (FC). Upon activation, ascidian sperm cells undergo a characteristic morphological change involving mitochondrial rounding and translocation. Activation is triggered by species-selective binding to FC N-acetylglucosamine, sperm integrin-dependent adhesion to FC surface and signaling via Rho GTPases to actin polymerization, and FC ligand activation of a G protein-coupled receptor (GPCR) that signals to myosin activation via myosin light chain kinase (MLCK). Here

we characterize crosstalk between the integrin- and GPCR-dependent signaling events responsible for MTL. Sperm cells were diluted, treated, fixed and scored for activation by use of light microscopy. Activation down the various pathways was achieved by using mAb 12G10 (integrin activator) and Mas-7 (GPCR activator). Sperm cells treated with Clostridium difficile toxin A (CdtxA, 125 nM) showed a decrease in both integrin and GPCR-dependent activation from 62.36±3.7% to 4.03±2.5% and 62.36±3.7% to 5.01±2.51%, respectively. Because the GPCR-dependent pathway showed some influence on the Rho family GTPases, the action of U-46619, which activates Gα12/13 the only G protein subunit that has a direct role on Rho family activation, was tested. It activated sperm (50.5±6.01), an action blocked by CdtxA (7.67±3.2%). To study the potential role of ROCK, we used the inhibitor Y-27632 (10μM), which blocked integrin-dependent activation (62.36±3.7% to 9.7±2.6%) but had no effect on GPCR-depndent activation. Activation by U-46619 was inhibited by Y-27632 (50.5±6.01 to 9.17%), suggesting that Gα12/13 is upstream of Rho-dependent ROCK activation. Lastly, we tested the placement of MLCK in the integrin- and GPCR- by using ML-9 (25 μM); ML-9 inhibited both pathways (62.36±3.7% to 10.36±5.03% and 62.36±3.7% to17.03±4.04%, respectively). This study supports the presence of crosstalk between the integrin- and GPCR-dependent pathways with ROCK activating MLCK and Gα12/13 activating RhoGTPases, respectively.

1393/B609

Tyrosine Phosphorylation Changes during Xenopus Sperm Acrosome Reaction and Fertilization.

R. Bates, B. J. Stith; Biology 171, University of Colorado Denver, Denver, CO

Artificial induction of the acrosome reaction is typically achieved through addition of calcium ionophores: ionomycin addition to Xenopus spermatozoa increased tyrosine phosphorylation in these proteins (as detected by Western blotting and Odyssey laser/infrared detection; Anti-Phosphotyrosine Mouse mAb 2C8, Calbiochem 525293): a 54 kDa protein (at 2.5 to 3 minutes after ionophore addition), in a 33 kDa protein (at 2.5 to 3 min, then again at 20 min), 25kDa (30 seconds to 2.5 min), and in a 47kDa protein (at 10, 30 and 45 minutes). A different calcium ionophore, A23187, induced only one response at 32kDa (at 5 minutes and then decreasing at 45 minutes). As we have noted that phosphatidic acid (PA) increases during the acrosome reaction and may induce the exocytosis event, we added PA to sperm and noted that there was a sharp increase in P-Tyr at 5 minutes for 54 kDa protein (at 5 min after PA addition), a 30 kDa band (2.5-20 min), and a 24 kDa protein (2.5 min). Bands at 54 kDa, 33 kDa and 25 kDa were removed, after Coomassie staining, for mass spectroscopy analysis on a MS/MS Qq-TOF (Qstar XL). Our current model for Xenopus fertilization involves sperm activation of phospholipase D1b which would increase phosphatidic acid (PA). We have found that PA specifically binds and activates Src tyrosine kinase, and we and others suggest that Src would then activate phospholipase C gamma. To confirm that Src is indeed activated at Xenopus fertilization, samples were probed for active PP60Src after fertilization (Invitrogen-Biosource, 44-660G, Hu PAb Y418). Three was an increase in Src tyrosine phosphorylation at 2 minutes after insemination.

1394/B610

The Acrosome from the Water Strider Aquarius Remigis Contains a Fluorescent Molecule That Assembles into a Tubular Matrix.

H. Miyata, D. J. Fairbairn, Y. Cheung, R. A. Cardullo; Biology, University of California Riverside, Riverside, CA

The acrosome of the water strider *Aquarius remigis* is unusually long (≈2.5 mm) and contains a tubular matrix. However the precise molecular composition of the acrosome remains unknown. Here, we report that the *A. remigis* acrosome contains an intrinsically fluorescent molecule with fluorescent properties consistent with that of a flavin-containing molecule. The intrinsic fluorescence appears first in the acrosomal vesicle of round spermatids and persists in the acrosome throughout spermiogenesis. Fluorescence Recovery After Photobleaching reveals that the fluorescent molecule exhibits unrestricted diffusion in the acrosomal vesicle of round spermatids, becomes progressively immobilized throughout spermiogenesis and is completely immobile in the acrosome of mature sperm. Taken together, these observations suggest a model in which flavin-containing molecules assemble into a tubular matrix during spermiogenesis. Supporting our model further, a tubular matrix isolated as a detergent/heat resistant fraction retains its intrinsic fluorescence. The fluorescent matrix is soluble under reducing conditions, suggesting that the matrix is stabilized by disulfide bridges, and contains a major protein band with an Mr of approximately 85 kD. Since flavin-containing molecules are capable of catalyzing various oxidation-reduction reactions, the fluorescent molecule may play a role in the formation of disulfide bridges to produce the matrix during spermiogenesis. Although the function and adaptive significance of this unusual molecular structure remain to be determined, the intrinsic fluorescence of the *A. remigis* acrosome will be a useful biomarker for following spermiogenesis and fertilization in water striders.

1395/B611

Roles of Sperm Arylsulfatase A (ASA) in Male Reproduction.

N. Tanphaichitr[1], H. Xu[1], K. Kongmanas[1], K. F. Faull[2], T. Rupar[3], l. Hermo[4]; [1]Chronic Disease, Ottawa Health Research Institute, Ottawa, ON, Canada, [2]Pasarow Mass Spectrometry La, NPL-Semel Institute, UCLA, Los Angeles, CA, [3]Biochemical Genetics Lab, CPRI, University of Western Ontario, London, ON, Canada, [4]Anatomy and Cell Biology, McGill University, Montreal, QC, Canada

We have previously shown that sperm surface ASA plays roles in mouse in vitro fertilization (IVF). To further validate the roles of ASA in vivo, ASA null male mice were employed. Mating experiments using wild type (WT) females were conducted for 5 months starting with 5-month old males. The average litter size of the ASA null males was significantly smaller than that of the WT (6.2 ± 2.4 vs 7.9 ± 1.3 pups/litter; $p < 0.05$). There were no apparent changes of mating behavior between knockout (KO) and WT males, and the numbers of litters produced by the two animal sets were similar (4.8 vs 5.5). At 5 months of age, the IVF rate and ZP binding ability of swim-up sperm from ASA-/- males and WT males were indifferent. However, when the animals were 8 months old, these fertilizing abilities were reduced in ASA null sperm (<20% control). Notably, testicular weight of the 8-month old ASA null males was ~50% of the WT testes, although developing spermatogenic cells of all stages and sperm existed in the testis of ASA-/-males both 5-month and 8-month old. However, apoptotic germ cells were more prominent in the 8-month old KO animals (219 apoptotic cells vs 10 apoptotic cells in the WTs/100 seminiferous tubule sections). This result may explain in part the decrease of the testicular weight of ASA-/-males. Since sulfogalactosylglycerolipid (SGG, aka seminolipid) is a natural substrate of ASA, we asked whether SGG was accumulated in sperm and testes of ASA-/-males. Our high performance thin layer chromatography (HPTLC) and densitometric analysis results revealed that there was ~20% increase of SGG in both

sperm and testes of 8-month old ASA-/- males, relative to the corresponding SGG levels in age-matched WTs. Also, a few extra glycolipid bands were observed in ASA depleted testes. One of these bands had a signal of m/z 778.4 m/z and contained a sulfate group, as shown by electrospray ionization mass spectrometry. The increased SGG amounts and the presence of the new sulfoglycolipid may have caused changes of lipid microdomains on the germ cell surface that affect the ZP binding ability of sperm, as well as development of spermatogenic cells in the testes.

1396/B612

Effect of Dimethylformamide on Freezing-thawing Boar Sperm.

S. Choi, M. Bae, E. Lee, S. Kim, M. Seo, Y. Lee, S. Choi, Y. Kwon, Y. Kim, H. Kim, J. Byun, J. Lee, Y. Hwang, D. Kim; Division of Biological Science, Gachon Univ. Medicine & Science, Incheon, South Korea

Due to high cryosensitivity of boar sperm, it is necessary to find effective cryoprotectant. Glycerol is one of the most used cryoprotectant in boar sperm cryopreservation. However, it gives to living cells osmotic damage, and especially in boar sperm, it shows more sensitivity to glycerol than other domestic animal. In other study, amides were used as a cryoprotectant in stallion sperm freezing. In this study, we evaluated boar sperm viability, sperm chromatin integrity and motility especially in amides extender and compared the efficacy of amides with glycerol. The concentration of original boar semen was controlled to 109/mL, and removed the seminal plasma with Hulsen solution. It is centrifuged for 3 min at 2400g, 15°C, and then diluted the pellet with the first extender, BF5. We added trehalose to BF5 for improve the post-thawed sperm quality. The second extender, addition of each glycerol and amides to BF5, was used to dilute the first extender and sperm. We froze the diluted sperm in liquid nitrogen. Extender with respective amides compared with extender including glycerol on viability, sperm chromatin structure integrity and motility after thawing. Percentage of motile sperm diluted with glycerol is as higher as that of stallion's study (61%). In extender with DMF, it displayed superior quality of boar sperm to extender including glycerol. In case of MF, it displayed slightly low quality of boar sperm compared to glycerol, but it indicates any significant difference. However, urea showed no significant difference of boar sperm quality. In conclusion, the amides, especially dimethylformamide affect positively on boar sperm quality and reduce cryoinjury from freezing process. It suggests that dimethylformamide is expected to substitute for glycerol as a cryoprotectant.
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1397/B613

Temporal Dynamics of Cannabinoid Effects on Sperm ATP Levels and Motility.

D. Morgan[1], C. H. Muller[2], N. A. Murataeva[1], S. E. Hastings[1], K. Mackie[1]; [1]Psychological and Brain Sciences, Indiana University, Bloomington, IN, [2]Urology, University of Washington School of Medicine, Seattle, WA

In the present study we have investigated the in vitro effects of two exogenous cannabinoid compounds, WIN 55,212-2 and delta-9-tetrahydracannabinol ($^{\Delta}$9-THC) on murine sperm ATP levels and motility. We find that treatment with either WIN 55,212-2 or $^{\Delta}$9-THC for 30 minutes reduces sperm ATP levels. The maximal effect of 5 μM WIN 55,212-2 (-35%) on sperm ATP is significantly less than the inhibitory effect of $^{\Delta}$9-THC at this concentration (-65%). When sperm were treated with 10 μM $^{\Delta}$9-THC, ATP levels were reduced 91% relative to untreated controls. The effect of WIN 55,212-2 on sperm ATP is attenuated in sperm lacking the CB_1 cannabinoid receptor. The non-psychoactive enantiomer, WIN 55,212-3, has no effect on sperm ATP levels. In contrast, the effect of $^{\Delta}$9-THC on ATP persists in sperm lacking CB_1 receptors suggesting that the inhibitory effect of $^{\Delta}$9-THC on ATP levels is not CB_1 mediated. The minimal concentration of $^{\Delta}$9-THC required to reduce sperm ATP is ~1-3 μM. In addition to inhibiting sperm ATP, 10 μM $^{\Delta}$9-THC also reduces the basal motility of wildtype sperm within 15 minutes of starting drug treatment. Basal motility was measured using computer-assisted sperm motility analysis and is maximally inhibited (-90%) after 30 minutes of treatment with 10 μM $^{\Delta}$9-THC. Interestingly, inhibition of basal motility with 10 μM $^{\Delta}$9-THC occurs when ATP levels are normal, suggesting that inhibition of motility by $^{\Delta}$9-THC is unlikely to be mediated by a reduction in ATP levels. While 300 nM $^{\Delta}$9-THC does not affect the percentage of motile cells this drug concentration does decrease the smoothed path velocity, straight line velocity, track velocity, and percentage of cells exhibiting progressive motility. These results suggest that $^{\Delta}$9-THC has biphasic effects on basal motility with high concentrations causing a profound decrease in the percentage of motile cells while lower concentrations lead to more subtle effects on sperm kinematics.

1398/B614

Sex Ratio Determination by Quantitative Real Time PCR Using Amelogenin Gene in Porcine Sperm.

M. Bae, S. Choi, E. Lee, S. Kim, Y. Kwon, M. Seo, Y. Lee, S. Choi, H. Kim, Y. Kim, J. Byun, J. Lee, Y. Hwang, D. Kim; Division of Biological Science, Gachon University of Medicine and Science, Incheon, South Korea

Sex determination is essential for application in assisted reproductive biotechnology (ART), mass production of desired sex livestock and conservation of breeds having excellent traits. Flow cytometric analysis is the most powerful technique to separate X-, Y-chromosome bearing sperm. However, this method requires revaluation by other sex determination systems in order to verify sorting efficiency. To reanalyze sex ratio of flow cytometric sorted-sperm, we used quantitative real-time polymerase chain reaction of the porcine amelogenin (AMEL) gene. The AMEL gene is present on both sex chromosomes in porcine species and the length and sequence of the noncoding regions differ between the two chromosome-specific alleles. A same set of primers can amplify a homologous sequence of AMEL gene in both X and Y chromosome. They are also more accurate than other Y-chromosome-specific primers targeting sex determining region Y (SRY) or male-specific repeat 1, which can not identify sequences in X chromosome. From combination of SYBR Green-DNA melt curve, frequency of X-bearing sperm was studied by approaching a relative quantification. There were two sets of primers. One targeted homologous sequence of AMEL gene in both X and Y chromosome. The other bound to sequences of AMEL gene in only X chromosome, which is deficient in Y-chromosome. Relative quantitation expressed as the starting fluorescence of specific sequences in X-bearing sperm normalized to that of homologous sequences in X-, Y-bearing sperm. The results of X- and Y-sorted semen samples analyzed by quantitative real-time polymerase chain reaction and by flow cytometric reanalysis showed no significant difference ($P > 0.05$). The result suggested that quantitative real-time

polymerase chain reaction-based method using AMEL gene in this studies is a reliable, accurate and rapid method for sex determination This work was supported by a grant (No.20070301-034-040-007-03-00)

1399/B615

Requirement for Sun1 in the Expression of Meiotic Reproductive Genes and piRNAs.

Y. Chi[1,2], L. I. Cheng[2], T. G. Myers[2], K. Jeang[2]; [1]Institute of Cellular and System Medicine, National Health Research Institutes, Zhunan, Taiwan, [2]National Institute of Allergy and Infectious Disease, National Institutes of Health, Bethesda, MD

The nuclear envelope (NE) interacts with the structural scaffold and participates in the architectural organization of chromosomes. The interaction of the NE-associated proteins with genomic DNA may contribute to the spatial arrangement of the chromosomes and their gene expression. Sun1 is an inner nuclear membrane (INM) protein that locates to telomeres and anchors chromosome movement in the prophase of meiosis. Here, we have created Sun1-/- mice and found that these mice are born and grow normally but are reproductively infertile. Sun1 presented stage-wise dynamic localization in meiosis, beginning in the nuclear periphery in spermatogonia, to telomeres during prophase I, and then at an acrosome-like locus on elongated spermatids. Detailed molecular analyses showed that Sun1-/- P14 testes cells are repressed for the expression of reproductive genes and have no detectable piRNAs. These findings point to an unrecognized role of Sun1 in the gene expression of coding and non-coding RNAs for gemetogenesis.

Stem Cells II (1400 – 1422)

1400/B616

The Drosophila Hindgut: Multiple Progenitors, No Active Stem Cells, and Robust Damage Response.

D. Fox[1, 2], A. Spradling[1, 2]; [1]Embryology, Carnegie Institution for Science, Baltimore, MD, [2]Howard Hughes Medical Institute, Baltimore, MD

Within an organ, cell proliferation programs differ during periods of organogenesis, organ maintenance, and damage-induced repair. How different organs regulate these distinct states is not well understood. Further, as tissue architecture and cell turnover differ drastically between organs, the need for stem cell based renewal also varies. Here, we investigate the cell proliferation program of the *Drosophila* hindgut during organogenesis, maintenance, and repair. Our findings contrast previous reports and suggest new models for hindgut proliferation. We find that proposed hindgut imaginal progenitors play a limited role in hindgut organogenesis, and that cells dispersed throughout the larval hindgut also act as progenitors. Furthermore, in striking contrast to the stem cell-based midgut, maintenance of the hindgut is limited and does not involve stem cells, similar to the proposed renewal strategy for the vertebrate pancreas. However, as in the pancreas and vertebrate liver, upon induced organ damage, the hindgut elicits a rapid and heightened proliferation response. These results suggest the *Drosophila* hindgut is an accessible model for study of non-stem cell based tissues during organ maintenance and damage-induced repair.

1401/B617

The Effects of Human Mesenchymal Stem Cell Growth Factors on Chick Embryo Neurons.

D. Coffey, J. Thompson; Biology, CSU San Bernardino, San Bernardino, CA

Adult-derived mesenchymal stem cells (MSC's) are found in a wide variety of tissues in the body. These cells have the ability, both in vitro and in vivo, to alternately remain in their un-differentiated state for numerous passages, or differentiate into cells consistent with those of their host tissues, or even cells of varying lineage. Bone marrow stromal cells, adipose-derived stem cells and umbilical cord cells have all shown the ability to differentiate into cells found in tissue quite different from their host tissue. Recent studies have indicated that, in addition to the differentiation potential of MSC's, these stem cells also produce various growth factors that influence other cells, both normal cells and endogenous stem cells. These growth factors act to recruit stem cells to the affected tissue and initiate the repair and replacement processes associated with normal healing. Our study investigates the effects of the growth factors produced by three different populations of MSCs on chicken retinal and brain neurons. We plated eight-day chick embryo neurons on poly-lysine coated tissue culture dishes and exposed them to the growth factors produced by three different types of MSCs. The growth factors were delivered via conditioned media produced by the MSCs. Conditioned media was collected from cultures of human bone marrow stromal cells, adipose-derived stem cells, and umbilical cord matrix cells. The effect of the conditioned media/growth factors on neurite length and number was quantified. Our results indicate increased neurite length and number in the presence of the mesenchymal growth factors from the MSCs.

1402/B618

Murine Bone Marrow and Mesenchymal Stem Cells Accelerate the Regeneration of Skin Wounds Treated with a Dermal Substitute.

N. B. Nardi[1], D. Leonardi[2], D. Oberdoerfer[1], R. C. Chem[2]; [1]Genetics, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil, [2]Universidade Federal de Ciências da Saúde de Porto Alegre, Porto Alegre, Brazil

Dermal skin substitutes are now a standard of care in burn treatment, and serve as a scaffold into which cells can migrate and repair the injury. The products have, however, poor take in an average of 20% of the cases, possibly due to limited feeding by the injury bed. Stem cells are known to have an important role in the regeneration of injured tissues. This work aimed to investigate if the addition of stem cells to lesions treated with a dermal substitute results in increased cellular and vascular density, in a murine model. Full-thickness wounds were created on the dorsum of adult C57BL/6 mice and treated with a dermal substitute (Integra). Total bone marrow cells (BM, 10E6/animal) or cultured bone marrow-derived mesenchymal stem cells (MSCs, 10E5/animal) from C57BL/6 GFP+ mice (green mouse FM131, M. Okabe, Osaka University, Japan) were transplanted between the wound and the dermal substitute. Control mice received only culture media. The animals (n=6/group) were sacrificed 4, 8, 12, and 16 days later and skin specimens were processed for histology, immunofluorescence and vascular density (immunohistochemistry with anti-CD31 antibodies). Statistical analyses included ANOVA and Duncan's post-hoc, with a significance level of 5% ($p<0.05$). GFP+ cells were observed mainly among the Integra fibers in mice treated with BM cells, and in the subjacent tissue in mice treated with MSCs, on days 4 and 8; fluorescent cells were rarely seen in the other two periods of analysis. Cellular

and vascular density were significantly higher in the groups treated with cells when compared to control groups, and in mice receiving MSCs when compared to BM. Theses differences were more clearly seen on days 4 and 8 for cell density, and on day 16 for vascular density. These results show that bone marrow, and particularly mesenchymal stem cells, may represent an important contribution to the treatment of burn wounds when dermal substitutes are employed.

1403/B619

Obtention of Adipocytes from Mesenchymal-Stem Cells Derived of Human Bone Marrow and Periostium.

A. Parra[1], L. F. Acevedo[2], A. Miliar[1], A. Salas[1], E. Gutierrez[3], G. Gutierrez[1], A. Dominguez[1]; [1]Biologia Molecular, Instituto Politecnico nacional, Mexico City, Mexico, [2]Immunology, Instituto Politecnico Nacional, Mexico City, Mexico, [3]Autonome National University of Mexico, Mexico City, Mexico

Evidences exist that stem cells are source of several cellular types and advantage is used in therapies to cure degenerative diseases. The different properties of nonembryonic stem cells (in special of mesenchymal-stem cells) are still unknown. In this work, we obtained bone marrow mesenchymal stem cell (MSC) and for another hand we also isolated stem cells derived from periostium (skin protection the surface of the bone) and we investigated the capability of these cells to differentiate in adipocytes. Methods and Results: MSCs were isolated and cultured from explants of tissue of bone marrow and periostium by separate and were maintained in culture medium during five days. Histological examination of both cultures revealed that the attach cells results in mesenchymal- stem cells. The differentiation was realized using an special medium of culture supplied to growth and differentiation factors. After 3 days, we supplied the medium with new growth factors and the cultures were maintained over 15 days. A specific staining for fat cells confirmed the presence of adipocytes. To confirm the cell differentiation, we used inmunohistochemical analysis and RT and real-time PCR to determine the presence of markers of adipocytes. Importantly, our data describe that mesenchymal-stem cells derived of human bone marrow and periostium could suffer differentiation and could be a new source for adipocytes. It further highlights the potential of MSCs cells and periostium as a model in the study of the regulation of physiologically adipose tissue, we describe a suitable method for this purpose.

1404/B620

Osteogenic Differentiation of Human Mesenchymal Stem Cells in Three Dimensional Matrices.

A. A. Sule[1,2], J. Shankardas[2], A. Nana[3], D. Dimitrijevich[1,2,3]; [1]Integrative Physiology, UNTHSC, Fort Worth, TX, [2]Graduate School of Biomedical Sciences, UNTHSC, Fort Worth, TX, [3]Department of Orthopedic Surgery, UNTHSC, Fort Worth, TX

Introduction: Significant bone voids cannot be repaired by unassisted regeneration. Autologous living grafts provide the ideal osteogenic habitat for regeneration but are hindered by serious disadvantages. Metallic foam implants are biocompatible, load bearing scaffold but cannot participate in bone remodeling events. The mineral scaffolds on the other hand (e.g. beta tricalcium phosphate, bTCP) participate in bone remodeling. **Purpose:** To demonstrate that porous bTCP scaffolds when impregnated with neutralized and gelled collagen type I solution (CT-I) provide a suitable environment for human mesenchymal stem cell survival, proliferation and osteogenic differentiation. **Materials and Methods:** Collagen type I foam, bTCP Preforms®, and tantallum foam discs were impregnated with CT-I containing fluorescently labeled human dermal fibroblasts (DFbs). After gelling at 37°C the cells were visualised using confocal microscopy. Treatment of the matrices with collagenase released the cells and allowed determination of their numbers. Experiments using human mesenchymal stem cells labeled with Cell Tracker Dyes® are in progress. **Results:** After impregnation collagen foam, bTCP, and tantalum absorbed 500%, 50%, and 10% of their weight in collagen gel respectively. Scanning electron microscopy demonstrated fibrillar appearance after gelling. Collagen populated with DFbs and was present throughout the matrices examined. Long-term viability (30 days), migration of DFbs in and out of the collagen-impregnated foam and cell alignment when the matrix was stretched were also demonstrated. **Conclusions:** All the porous matrices examined absorb and retain collagen type I as a fibrillar hydro-gel. The cells introduced suspended in the collagen solution were randomly distributed throughout the impregnating gel matrix. Long-term cell survival in these three-dimensional matrices will allow time for in situ differentiation. Free migration of the cells into and out of the matrix, demonstrated its cytocompatible nature and their alignment in response to external forces suggest potential for behavior appropriate for bone development. Taken together these observations suggest that the strategy being tested will be host compatible.

1405/B621

Mouse Adult Kidney-Derived Mesenchymal Progenitor Cells Promote Functional Recovery of an Ischemic/Reperfusion Kidney.

H. Lin[1], P. Lee[2], S. Jiang[3], M. Tang[1,2]; [1]Physiology, National Cheng Kung University, Tainan, Taiwan, [2]Clinical Medicine, National Cheng Kung University, Tainan, Taiwan, [3]Molecular Biology, Academia Sinica, Nankang, Taipei, Taiwan

Acute renal failure is an important problem in clinic and acute tubular necrosis accounts for the most common cause of acute renal failure in hospital. After injury, the kidney undergoes a regenerative response to recover renal function. Three possible sources of new tubular cells are considered to replace damaged epithelial cells: less injured tubular cells, bone marrow-derived cells or resident renal stem cells. In our preliminary study, a unique population of cells that behave in a manner consistent with renal stem cells was isolated from GFP-targeted Myh9 transgenic adult mice, named mouse kidney progenitor cells (MKPC). Features of MKPC included self-renewal more than 100 passages without evidence of senescence; expression of Pax-2, Oct-4, vimentin, α-smooth muscle actin, CD 29 and S100A4 but not SSEA-1, CD133, c- kit, E-cadhrein, CD45R, CD34. MKPC exhibited pluripotent differentiated ability as demonstrated by the ability of differentiation into endothelial cells and osteocyte. More importantly, MKPC could protect ischemia reperfusion-injured mice from renal function and structure deterioration. To further explore the possible underlying mechanism, we employed 3-D collagen gel culture system. MKPC cultured in collagen gel developed capillary like network with positive staining for von Willebrand factor (vWF). When M1 (mouse collecting tubule) cells were cocultured with MKPC, M1 cells developed tubular-like structure with numerous branch. We also found that MKPC expressed GDNF mRNA as determined by reverse transcription-polymerase chain reaction (RT-PCR). Taken these data together, we suggested that MKPC maybe through secreting GDNF to elicit paracrine effect or by promoting angiogenesis to improved renal function.

1406/B622

Neural Stem Cells Engineered to Express L1 under the Control of the Human GFAP Promoter Promote Functional Recovery After Transplantation in a Mouse Spinal Cord Injury Model.

J. Xu, Y. Cui, G. Hargus, C. Bernreuther, M. Schachner; Zentrum für Molekulare Neurobiologie Hamburg, Hamburg, Germany

The transplantation of neural stem/progenitor cells (NSPCs) is a promising approach for cell replacement therapy after spinal cord injury. However, grafted neural stem cells differentiate mainly into astrocytes with only a minor fraction differentiating into neurons or oligodendrocytes. The immunoglobulin superfamily adhesion molecule L1 plays an important role in the development and regeneration of the central and peripheral nervous systems and improves cell survival, neurite outgrowth and neuronal differentiation. To target NSPCs of the predominant astrocytic differentiation pathway with the ability of L1 to promote neuronal differentiation, cells were isolated from the brains of embryonic mice ectopically expressing L1 under the control of the human GFAP promoter (hGFAP-L1 NSPCs). We found L1 expressed in radial glial cells and astrocytes differentiated from these hGFAP-L1 NSPCs, which showed increased neuronal differentiation, enhanced migration, and decreased astrocytic differentiation when compared to wild type NSPCs in vitro. Mice injected with hGFAP-L1 NSPCs into the compression-lesioned spinal cord showed better locomotor functional recovery as compared to mice that had been injected with wild type (WT) NSPCs or PBS as vehicle control. Morphological analysis revealed increased neuronal differentiation and migration of transplanted cells as well as increased numbers of endogenous catecholaminergic (tyrosine hydroxylase [TH]+) axons caudal to the lesion site in the hGFAP-L1 NSPC group when compared to the WT and PBS groups. Furthermore, soma size and synaptic coverage of host motoneurons increased caudal to the lesion site in the hGFAP-L1 NSPC group in comparison to the other groups. These findings indicate that hGFAP-L1 NSPCs are capable of improving functional recovery in a syngeneic transplantation paradigm by modulating regenerative processes in the injured spinal cord.

1407/B623

Cell Division Defects in a New Mouse Model of Dominant Microcephaly.

D. L. Silver[1], D. M. Larson[1], D. Watkins-Chow[1], N. Gaiano[2], W. Pavan[1]; [1]GDRB, NHGRI/NIH, Bethesda, MD, [2]Institute for Cell Engineering, JHMI, Baltimore, MD

Microcephaly is characterized by reduced brain size and is largely attributed to defects in neuronal progenitor cell (NPC) division, although the genetic basis for this disease is not completely understood. From a genome-wide mouse mutagenesis screen we identified a new semi-dominant microcephaly mutant called *Oreo (Or)*. In neocortices of *Or+/-* embryos, late-born neuronal layers were disorganized and markedly reduced. Of note, there was also a dramatic reduction in intermediate neuronal progenitors, indicating that the reduced neurons in *Or+/-* animals is likely due to loss of this neuron producing cell population. At the onset of neurogenesis, *Or+/-* embryos had ectopic production of neurons, many of which underwent apoptosis, as well as altered orientation of the mitotic spindle of apically dividing NPCs. The division plane of NPCs may be associated with whether NPCs undergo symmetric divisions to replenish the NPC population or asymmetric divisions to produce both NPCs and neurons. Consistent with a cell division defect, Mefs from *Or+/-* embryos exhibited increased frequency of polyploidy and aneuoploidy, as assessed by metaphase and SKY analyses. Together our results indicate that the microcephaly in *Or+/-* animals is due to alterations in cell division; this in turn results in defective NPC maintenance, fewer neurons, and neuronal apoptosis.

1408/B624

Comparison of Osteogenesis of Osteogenic Cells within 2D and 3D Culture Systems.

W. Liu[1], W. Lau[1], Q. Liu[1], W. Sun[2]; [1]3D Biotek, North Brunswick, NJ, [2]Department of mechanical Engineering, Drexel University, Philadelphia, PA

The physiology of a variety of different cell types has been shown to be remarkably different within 3-dimentional (3D) environment compared to 2-dimentional (2D) monolayer culture environment. 3D scaffolds made from synthetic polymers may offer various structure and mechanical features to match tissue specific applications. Therefore, they are drawing tremendous attention in bone tissue engineering field. Polycaprolactone (PCL) is a biodegradable polymer that has been used to make 3D scaffolds for various tissue engineering applications. However, there has not yet been a report comparing osteogenesis of osteoprecursor cell lines within 2D and 3D PCL culture system. The objective of this study was to compare the growth pattern of osteoblast within 2D and 3D PCL culture systems. Mouse 7F2 osteoblast cell line was seeded at the same cell density (cell number/cm2 surface area) on 2D polystyrene (PS) culture plate, 2D polycaprolactone (PCL) film, and 3D PCL porous interconnected scaffold, respectively. Osteogenic differentiation was induced through culture media supplemented with L-ascorbic acid and β-glycerophosphate. Quantitative and qualitative assays of osteogenic differentiation were conducted at multiple time points during 4 weeks of in vitro culture period. It has been found that osteocalcin secretion and alkaline phosphatase (ALP) activities were significantly higher in 3D PCL culture system than in both 2D PS and 2D PCL film systems ($P < 0.05$, t-test). Von Kossa staining showed more extensive mineralization of 7F2 cells on 3D PCL scaffold than in 2D PS and 2D PCL monolayer culture. The Scanning Electron Microscopy (SEM) results further showed a more discernable bone-extracellular matrix formation within 3D system compared with 2D culture. In conclusion, our results demonstrate that the osteogenic differentiation of osteoblast is enhanced in a 3D PCL culture system as compared to 2D culture environment.

1409/B625

A Novel Animal Model Displaying GFP Reporter in Adult Neural Stem/Progenitor Cells.

C. Sung, N. Gruzdeva, A. Li, J. Chuang; Ophthalmology/Cell & Dev. Biology, Weill Cornell Medical College, New York, NY

Stem cell research in the central nervous system holds great promise for the development of novel therapies for brain damage and other human diseases. In the dentate gyrus of the hippocampus, new neurons continue to be derived from resident stem/progenitor cells throughout our lives and functionally participate in learning and memory. Our lab has previously shown that Tctex-1, previously recognized as a light chain of cytoplasmic dynein, is selectively enriched in proliferating progenitors, but not in the mature granule cells and astrocytes, in the adult dentate gyrus (Dedesma, JCN 496: 773-786.2006). We thus sought for genomic sequences specifying Tctex-1 expression in the adult neural stem/progenitor cells. “Promoter bashing” experiments were first carried out in developing neocortex by ectopically expressing GFP

reporters driven by various upstream and/or intronic sequences of Tctex-1 using in utero electroporation method. A reporter construct encompassing ~9.6-kb genomic DNA fragment that directs specific GFP expression in neural stem/progenitor cells in the embryonic brains was subsequently subjected to generate transgenic mice. Confocal microscopic examination of mouse brains derived from three independent transgenic lines showed that GFP and endogenous Tctex-1 shared similar subgranular zone distribution. Colocalization studies with NeuN, GFAP, Nestin, and Doublecortin suggested that the GFP specifically mark neural progenitors in adult brains. We are currently characterizing the GFP expression in other tissues of these reporter mice.

1410/B626

Neural Lineage Differentiation of Three Dental Progenitor Cell Populations.

L. L. Li[1], Y. Ito[2], T. Diekwisch[2]; [1]NCHS, Naperville, IL, [2]UIC, Chicago, IL

The human central nervous system (CNS) has a limited capacity for self-repair. As a result, stem cell based approaches for the regeneration of neural tissues hold great promise as a therapy for neurodegenerative diseases. Dental progenitor cells are a unique resource for neural tissue repair and regeneration because of the accessibility and autologous potential of tissues derived from third molar teeth and because of their ability to self-renew and differentiate into multiple lineages. In order to determine the ability of dental progenitor populations to form neural tissues, dental follicle (DF), dental pulp (DP), and periodontal ligament (PDL) cells were subjected to neurosphere-forming culture conditions and neuronal and glia cells were selected. The selected cells were either cultured on laminin-coated cover slides or mixed with collagen gel. The cell-collagen constructs were cultured in vitro or implanted subcutaneously into nude mice. Differentiation of progenitor populations into neurons, astrocytes and oligodendrocytes was evaluated using immunohistochemistry, real time RT-PCR and Western blot analysis. DF, DP and PDL cells formed neurospheres at frequencies of 0.2%, 0.4% and 0.02%, respectively. Dental progenitor-derived neurospheres expressed nestin and Sox10, based on immunostaining and RT-PCR analysis. Under differentiation induction conditions, DF-, DP- and PDL-derived spheres formed β3-tubulin-positive neurons, GFAP-positive astrocytes. In comparison, DF- and DP-derived spheres showed a higher potential to differentiate into neuron-like cells while PDL-derived spheres preferentially differentiated into astrocytes. Histological analysis and immunostaining demonstrated that sphere-forming cells actively proliferated in collagen gels, differentiated into neurons astrocytes and oligodendrocyte in three dimensions, and expressed neuronal or Schwann cell-specific markers. Together, these findings suggest that DF, DP and PDL progenitor cells are capable of differentiating into neural lineages and might harbor the potential to be used in the regeneration of neural tissues. Funding by NIDCR grant DE015425 to TD is gratefully acknowledged.

1412/B628

Stem Cell Antigen-1 Regulates the Expansion of Hematopoietic Stem Cell Subpopulations Following Bone Marrow Injury.

H. C. Kwan[1], S. Khalifian[2], C. Ritner[1], H. S. Bernstein[1,2,3]; [1]Cardiovascular Research Institute, University of California, San Francisco, CA, [2]Pediatrics, University of California, San Francisco, CA, [3]Institute for Regeneration Medicine, University of California, San Francisco, CA

Stem cell antigen-1 (Sca-1;Ly6A) is a GPI-anchored protein widely used as a cell surface marker for hematopoietic stem cells (HSCs). Our laboratory identified Sca-1 as a marker of activated myoblasts, and subsequently demonstrated both *in vitro* and *in vivo* that Sca-1 regulates myoblast proliferation, suggesting an important function for Sca-1 in skeletal muscle homeostasis, and a potential role in muscle stem cell maintenance. Since Sca-1 influences the tempo of myoblast proliferation following injury, we hypothesized that it may also affect stem cell activation and self-renewal. To study this, we adopted the model of 5-fluorouracil (5FU)-stimulated bone marrow "injury" and HSC activation. We injected 150 mg/kg 5FU intra-peritoneally into 8-10 week-old BALB/c Sca-1$^{+/+}$ and Sca-1$^{-/-}$ female littermates, harvested bone marrow from the femurs and tibias of treated animals at 72 hrs post-injection, and analyzed the HSC compartment by flow cytometry. Bone marrow of 5FU-injected Sca-1$^{+/+}$ animals showed a 1.5-fold increase in total cell count at 72 hrs, compared to no change in cell numbers harvested from bone marrow of similarly treated Sca-1$^{-/-}$ animals. This suggests that either the HSC response in the absence of Sca-1 is different from that seen in muscle, or that the absence of Sca-1 accelerates HSC mobilization from bone marrow into the peripheral circulation upon activation. The Lin$^-$ c-kit$^+$ and Lin$^-$ c-kit$^-$ subpopulations both increased 6-fold in Sca-1$^{+/+}$ bone marrow 72 hrs post-injection. Sca-1$^{-/-}$ bone marrow, however, demonstrated a 6-fold increase in Lin$^-$ c-kit$^+$ cells but a lesser increase (3-fold) in the Lin$^-$ c-kit$^-$ subpopulation at the same time point. Again, additional studies are needed to distinguish between decreased proliferation versus increased mobilization of this HSC subpopulation in the absence of Sca-1. These studies suggest, however, that the HSC response to injury is altered in the absence of Sca-1, and provide a model for interrogating Sca-1-mediated mechanisms for stem cell activation, mobilization and self-renewal.

1413/B629

Transcriptional Regulation of Injury-Induced Bone Marrow-Derived Cell Recruitment.

K. Mace[1,2], T. Restivo[2], J. L. Rinn[4], A. Paquet[3], H. Chang[4], D. M. Young[2], N. Boudreau[2]; [1]Life Sciences, University of Manchester, Manchester, United Kingdom, [2]Surgery, University of California, San Francisco, CA, [3]Medicine, University of California, San Francisco, CA, [4]Program in Epithelial Biology, Stanford University, Stanford, CA

The regulated recruitment and differentiation of pluripotent bone marrow-derived cells to sites of injury is critical for efficient wound healing. Previously we demonstrated enforced expression of *HOXA3* accelerated wound healing and promoted angiogenesis in the *Lepr[db]* mouse model of type 2 diabetes. In this study, we have used GFP+ bone marrow chimeras to investigate the effect of *HOXA3* expression on recruitment of bone marrow-derived cells to wounds. We hypothesized that the enhanced neovascularization induced by *HOXA3* is in part due to a modulation of the recruitment and/or differentiation of bone marrow-derived cells to the wound. We found that diabetic mice treated with *HOXA3* did have both a significant increase in early endothelial progenitor cells (GFP+CD34+CD133+) mobilized from the bone marrow, as well as more mature endothelial progenitor cells (GFP+CD34+) cruited to the wound than controls. Interestingly, however, we found *HOXA3*-treated mice had significantly less inflammatory (GFP+CD45+) cells in the wound than controls, suggesting that in addition to promoting angiogenesis, HOXA3 may function in the wound to suppress inflammation. Furthermore, microarray analyses to identify downstream target genes of *HOXA3* revealed that *HOXA3* function results in upregulation of genes that enhance recruitment of endothelial progenitor cells while repressing pro-inflammatory gene expression within the wound. Altogether our data suggests that *HOXA3* may

enhance neovascularization and cutaneous wound repair in the diabetic environment by regulating genes that selectively promote stem/progenitor cell recruitment while suppressing inappropriate inflammation.

1414/B630

Block Myostatin via AAV2-Delivered Myostatin Propeptide in Dystrophic Muscle Improves Dystrophic Pathology and Muscle Cell Transplantation.

J. Zhu[2,3], J. Ma[4,2], C. Qiao[4], J. Li[4], Y. Li[1,2], X. Xiao[4], J. Huard[1,2]; [1]Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA, [2]Stem Cell Research Center, Children's Hospital of Pittsburgh of UPMC, Universtiy of Pittsburgh, Pittsburgh, PA, [3]Bioengineering, University of Pittsburgh, Pittsburgh, PA, [4]UNC School of Pharmacy, University of Pittsburgh, Chapel Hill, NC

Myostatin (MSTN) is a potent negative regulator of muscle growth. We have found that like TGF-β1, MSTN also contributes to the formation of fibrosis in injured skeletal muscle. In the current study we utilized an adeno-associated viral vector carrying the MSTN propeptide gene (AAV-MPRO), an inhibitor of MSTN, to specifically inhibit the action of MSTN in the skeletal muscles of mdx/SCID mouse, a model of muscular dystrophy with immune-deficiency. Our objective was to investigate whether AAV-mediated MPRO attenuated the severity of muscular dystrophy and improved the success of cell transplantation. In the current study we injected AAV-MPRO/GPF particles in 50 µl of PBS into the gastrocnemius muscles (GMs) of mdx/SCID mice (4 weeks of age) 4 weeks prior to muscle progenitor cell (MPC) transplantation (300,000 cells per GM). Two weeks after cell transplantation, mice were sacrifice, and muscle samples were harvested and cyrosectioned. Histology (H&E and Masson's trichromine stain) and immnunohistochemstry (dystrophin) were performed. Our results revealed that MPRO improved the dystrophic pathology of mdx mice by promoting muscle growth and reducing collagen deposition. After AAV2-MPRO transfer, a significant increase in muscle weight was observed in comparison to the AAV2-GFP transduced control. Moreover, the mean diameter of the muscle fibers in the AAV2-MPRO transduced muscles was significantly larger than that of AAV2-GFP transduced muscles. And the number of degenerative foci was decreased in the former muscles. Masson's trichrome stain indicated a significant reduction of fibrous scar tissue in the AAV2-MPRO transduced GMs. Normal MPCs injected into AAV2-MPRO transduced dystrophic muscles surpassed the regeneration capacity of cells injected into AAV2-GFP transduced control muscle, evidenced by significantly more dystrophin-positive myofibers in the former. In conclusion, AAV2-MPRO ameliorated dystrophic pathology of mdx/SCID mice, and improving the milieu in the host dystrophic muscle prior to cell transplantation is an alternative approach to enhance the efficiency of cell transplantation.

1415/B631

"Cardiac-Like" Nuclear Ca2+ Oscillations Induce Somatic Stem Cells to Differentiate into Cardiomyocytes.

P. Anderson[2], B. Muller-Borer[3], G. L. Esch[1], N. N. Malouf[1]; [1]Department of Pathology and Laboratory Medicine, School of Medicine at UNC Chapel Hill, Chapel Hill, NC, [2]Department of Pediatrics, Duke University Medical Center, Durham, NC, [3]Department of Internal Medicine, Brody School of Medicine at ECU, Greenville, NC

The epigenetic regulation of adult derived somatic stem cell differentiation into cardiomyocytes is not known. We have used a rat clonal adult liver-derived stem cell line (WB F344) and human mesenchymal stem cells (hMSC), as prototypical somatic stem cells, to examine the underlying basis for their acquisition of a cardiomyocyte phenotype. These stem cells acquire a well differentiated cardiomyocyte phenotype in vivo in the hearts of nude mice and ex vivo in co-cultures adjacent to contracting neonatal rat cardiomyocytes. We found that the first event in the sequential process that results in the acquisition of this cardiomyocyte phenotype is formation of Connexin 43-derived shared functional gap junctions between the cytoplasm of the stem cells and that of juxtaposed contracting cardiomyocytes. A signal from the cardiomyocyte, transmitted across these channels, is translated into stem cell nuclear Ca2+ oscillations ([Ca2+]n), that are synchronous with the Ca2+transients in the adjacent cardiomyocytes. These De Novo [Ca2+]n oscillations are associated with up-regulation in expression of Ca2+/calmodulin dependent cascade effectors and activation of a cardiac transcriptional gene program in the stem cell. We have identified preferential expression of the IP3 receptor 1 (IP3R1) in the stem cell nuclear membrane and found that IP3R1 inhibition eliminated the [Ca2+]n oscillations and differentiation of stem cells into cardiomyocytes. We furthermore found that silencing the expression of Connexin 43 in the stem cell with siRNAs or chemical mutagenesis aborted the process of acquisition of the cardiac phenotype by the stem cell. Collectively, our results suggest that "cardiac-like" [Ca2+]n oscillations in naïve uncommitted adult derived stem cells induce through a Ca2+ signaling cascade their differentiation into cardiomyocytes.

1416/B632

S100B Activates Muscle Satellite Cells via RAGE Engagement.

F. Riuzzi, G. Sorci, R. Donato; Experimental Medicine and Biochemical Sciences, University of Perugia, Perugia, Italy

We reported that treatment of high-density myoblasts and isolated satellite cells in differentiation medium (DM) with pM amounts of S100B caused inhibition of differentiation and apoptosis, and stimulation of proliferation via receptor-mediated stimulation of the mitogenic MEK-ERK1/2 and inhibition of the promyogenic p38 MAPK. However, a short-term (24 h), but not long-term treatment of low-density myoblasts with S100B resulted in enhanced myogenic differentiation via the simultaneous activation of p38 and ERK1/2. These differential effects of S100B depended on engagement of RAGE (receptor for advanced glycation end products) in low-density myoblasts and of bFGF receptor (FGFR) 1 in high-density myoblasts. We now show that intramuscular injection of S100B results in activation of muscle satellite cells (SCs) and stimulation of their proliferation, as inferred by the increased numbers of Pax7-positive cells and BrdU-positive cells, respectively, compared with sham-injected muscles. By contrast, no such effects could be observed upon injection of S100B into muscles of RAGE null mice. S100B is expressed in mature myofibers and is present in crushed muscle extract (CME). Blocking S100B in CME with an S100B neutralizing antibody results in reduced ability of CME to stimulate myoblast proliferation. Also, a short-term (24 h) treatment of low-density myoblast cultures with S100B on day 4 after their switch to DM results in enhanced myotube formation and myotube hypertrophy likely due to S100B-induced activation of quiescent myoblasts and stimulation of their proliferation. Indeed, S100B accelerates the proliferation of myoblasts upon their switch from quiescence medium to DM. Collectively, our results suggest that S100B released from damaged muscles might activate SCs via RAGE engagement. However, S100B enhanced the proliferation of RAGE null primary myoblasts, an effect that was

blunted in the presence of an FGFR1 neutralizing antibody. Thus, S100B might activate muscle SCs via RAGE engagement and expand activated SCs via FGFR1 engagement.

1417/B633

Role of Necdin in Skeletal Muscle Regeneration.

P. Pessina[1], S. Francois[1], G. Cossu[2], R. Meneveri[1], S. Brunelli[2,1]; [1]Experimental Medicine, University of Milano Bicocca, Monza, Italy, [2]Stem Cell Research Institute, S. Raffaele Hospital, Milan, Italy

Mesoangioblasts are vessel-associated stem cells that have been shown to be ideal candidates for stem cell therapy of muscular dystrophies. However, despite of the identification of mesoangioblasts as potential source of skeletal muscle, detailed studies on their molecular pathways are needed to improve their ability to act as pool of resident stem cells. We believe that Necdin may be a valid candidate molecule to such intent. Necdin is a member of MAGE protein family and it is expressed during neurogenesis but also in both adult and developing skeletal muscle suggesting its relevant role in this tissue. Our group provided the first evidence that Necdin mediates skeletal muscle regeneration by promoting myoblasts survival and differentiation. It would be of great interest if this role could also be exploited in mesoangioblasts. Therefore we are trying to investigate this aspect, by isolating and characterizing adult muscle-derived mesoangioblasts from wild-type, Ndn-/- mice and mice over expressing necdin under a muscle specific promoter (MlcNec). We are studying their proliferation, response to apoptotic stimuli and their myogenic differentiation potential in vitro. By FACS analysis, these polyclonal culture show the same expression profile of stem cells markers. When skeletal-muscle differentiation is induced by coculturing mesoangioblasts with C2C12 myoblasts, they are able to fuse into hybrid myotubes. Moreover, a constitutive overexpression of Ndn by lentiviral infection of wt cells increases the differentiation ability of mesoangioblasts. In parallel, we are injecting necdin over-expressing mesoangioblasts and GFP expressing mesoangioblasts in the muscle of alpha-sarcoglycan KO mice and we are evaluating their contribution to muscle regeneration in vivo, by measuring the expression of GFP and alpha-SG respectively, by Real-Time, immunofluorescence and Western Blot. These data will be compared with those obtained in alpha-SG KO mice treated with mesoangioblasts from Ndn-/- mice. Understanding the molecular mechanism of the effect of necdin on regeneration and designing tools to boost this process, may be a crucial step towards the optimization of cell therapy by mesoangioblasts or other stem cells.

1418/B634

Differentiation Control of Murine Embryonic Stem Cells to Chondrocyte Lineage by Hydrostatic Pressure.

S. Horo[1], T. Akimoto[2,3], H. Zhang[3], K. Furukawa[1], T. Ushida[2]; [1]Graduate school of Engineering, The University of Tokyo, Tokyo, Japan, [2]Center for Disease Biology and Integrative Medicine, Graduate school of Medicine, The University of Tokyo, Tokyo, Japan, [3]Consolidated Research Institute for Advanced Science and Medical Care, Waseda University, Tokyo, Japan

We have reported that hydrostatic pressure, a mechanical stress that is generally applied to chondrocytes, was able to redifferentiate chondrocytes that have lost its primary phenotype. In this study, we examined whether hydrostatic pressure promotes chondrogenic differentiation of pluripotent murine embryonic stem (ES) cells. Murine ES cell were aggregated and formed spheroids by rotation culture without leukemia inhibitory factor (LIF). After 5 days in rotation culture, the formed embryoid bodies (EBs) were subjected to 20 MPa hydrostatic pressure for 60 minutes and plated onto gelatin-coated culture dishes. After additional 7 days culture, total RNA was extracted and gene expression patterns were determined by real-time PCR analysis. As a result, the expressions of chondrocyte-specific marker genes including collagen type 2 and transcription factor Sox9 were significantly increased compared to the control conditions. On the other hand, the expression of Oct-4, an undifferentiated marker, was significantly decreased compared to the control condition. These results suggest that hydrostatic pressure has a potential to control ES cells to chondrocyte lineage *in vitro*.

1419/B635

Derivation of Porcine Embryonic Stem (pES) Cell-like Cells.

K. Thansa, P. Fisher, K. Campbell; Animal Physiology, the University of Nottingham, Leicestershire, United Kingdom

The aim of this study is to produce pES-like cell lines by modifying isolation and culture conditions. In vivo porcine blastocysts at day 6-8 were assigned into two groups distinguished by their inner cell masses (ICMs) and epiblasts. In each group, intact blastocysts and isolated ICMs or epiblasts were cultured in either KO4bh or DM40bh media on mitotically inactivated mouse embryonic fibroblasts as previously described (Thansa, Fisher and Campbell, 2007). Two morphologically distinct of pES-like cells were isolated from epiblasts (pESA/B) whilst, no cell lines were established from ICMs. pESA-like cells were observed as individual small round cells with one, two or multiple nucleoli and having a high nucleocytoplasmic ratio, pESB-like cells formed domed-like colonies. pESA-like cells stained both negative and positive for alkaline phosphatase, but pESB-like cells stained 100% positive. Although, immunofluorescence staining of Octamer-4 (OCT-4) and nanog seemed not to stain positive with nuclei of these pES-like cells, all pluripotent gene markers such as OCT-4, nanog, sex determining region Y (SRY)-box2 (SOX-2), RNA exonuclease1 homolog (REX-1) and developmental pluripotency associated3 (DPPA-3) were detected similarity to mouse ES (mES) cells (Brons et al., 2007; Tesar et al., 2007). The abilities of pESB-like cells to form neurons, smooth muscles and hepatocytes were conducted in feeder-free culture system, they stained positive with nestin, α-smooth muscle actin and α-fetoprotein, respectively. In addition, pESB-like cells contained mRNAs specific of the three germ layer cells such as nestin, α-smooth muscle actin, smooth muscle myosin, α-cardiac actin, transthyretin, α-fetoprotein, albumin and HGF1-β. In conclusion, pESB-like cells possibly have the potential to be stable ES cells isolated from early epiblast origin as mES cells.

1420/B636

Growth Senescent Mammary Epithelial Cells Form Competent Stem/Progenitor Cell Niches.

C. Boulanger, G. Smith; MBTL, NIH, Bethesda, MD

Upon serial transplantation into epithelium-divested mammary fat pads, mouse mammary epithelium consistently shows growth senescence beginning around the 4th transplant generation. This occurs at local sites within the mammary outgrowth and not as a global phenotype. To determine whether this loss of growth potential is due to stem/progenitor cell exhaustion or the inability of the local mammary epithelial

niche to support stem/progenitor cell self-renewal, we combined 100,000 (100K) growth senescent mammary epithelial cells (MEC) 2:1 with WAP-Cre/Rosa26R-lacZ-derived neural stem cells (NSC) and inoculated them into cleared mammary fat pads of hosts that were subsequently bred and allowed to lactate. Following mammary gland involution, the transplanted mammary glands were whole mounted and reacted with X-gal to determine the presence of mammary cells derived from NSC's. This is a result of the NSC stem/progenitor cells filling competent niches from the senescent mammary populations. Whereas, no positive mammary outgrowths (0/10) were obtained when 100,000 WAP-TGFβ1 MEC were transplanted alone, 6/10 outgrowths filling 25-70% of the fat pad were found in mice inoculated with the 2:1 (100K/50K) mixture of MEC and NSC. Secondary outgrowths from these chimeras also grew in cleared mammary fat pads indicating the support of continued stem/progenitor MEC self-renewal during chimeric growth/expansion. We conclude that stem/progenitor exhaustion and NOT stem cell niche decay is the cause for growth cessation. This implies that the cells that make up the mammary niche can remain active in growth incompetent tissue and can accept and support stem/progenitor cells from other organ sites. The activity of these niche-comprising cells may play an important role in the generation of neoplasia in adult organs.

1421/B637

Characterization of Parasitic Stem Cells in the Invertebrate Chordate Botryllus schlosseri.

M. Roux; [1]School of Medicine, Stanford University Hopkins Marine Station, Pacific Grove, CA, [2]Institute for Stem Cell Biology and Regenerative Medicine, Stanford University, Palo Alto, CA

Developmental programs that control cell proliferation and differentiation during growth and regeneration are mediated by a long-lived pluripotent population of stem or progenitor cells. Although these cells control the most fundamental aspects of biology, the cellular and molecular mechanisms that define them and control them are not well understood. The colonial ascidian, Botryllus schlosseri, provides a unique model to study chordate stem cell biology. Regeneration is a major part of the life history of Botryllus: in a highly coordinated developmental process, Botryllus adults regenerate themselves, including all somatic tissues and the germline, every week. Botryllus is the closest related species to the vertebrates with this regenerative ability, and we have developed the methodology to enrich the stem cells responsible. In addition, naturally occurring fusion events between individuals these cells can actually be parasitic, and upon transplantation, either naturally (the individual free-living forms of the organism can undergo vascular fusion to form chimeric individuals) or experimentally, can take over and replace both germline and somatic tissues of another individual in an event called germline cell parasitism (gcp) or somatic cell parasitism (scp) respectively. This parasitic ability is not "random", it is genetically-determined, therefore fundamental aspects of stem cell biology, such as self-renewal capacity, homing or expansion and differentiation kinetics must underlie the ability of a stem cell of one genotype to out compete a stem cell of another genotype. The goals of this study are to characterize and further enrich these stem cells using molecular markers and determine the role of these genes in gcp and scp. We have characterized the developmental expression patterns of several stem cell markers temporally using qPCR and spatially by in situ hybridization. We are also in the process of using FACS to isolate positive populations for further molecular analysis and transplantation assays.

1422/B638

Induced Pluripotent Stem Cell Based Models of Human Disease.

J. T. Dimos; Izumi Bio, Inc., Mountain View, CA

Treatments for many degenerative diseases are lacking because the cell types affected are not available for research; either they have already been lost at the time of diagnosis, or they cannot reliably be isolated from patients. Reprogramming technologies enable use of primary human cells to generate cell culture based models of degenerative disease. Induced pluripotent stem (iPS) cells could be used to produce an inexhaustible supply of particular cell types using directed differentiation approaches. iPS cell lines generated directly from a patient would contain the precise genetic constellation leading to disease in that patient. These patient-specific iPS cells could then be differentiated into disease-relevant cell types analogous to those lost to degeneration. These patient-specific disease relevant cells have high utility for investigation of early disease mechanisms and drug screening. As proof-of-principle, we have shown that large quantities of human motor neurons could be produced, in a robust and scalable manner, from iPS cells generated from a patient with amyotrophic lateral sclerosis (ALS), and that iPS cell derived motor neurons survive in long term culture. Our current efforts are aimed at generating and differentiating patient-specific iPS cells on an industrial scale to establish and interrogate cell culture based models in statistically rigorous ways for a number of common diseases, including neuromuscular pathologies. These cellular models of human disease should allow us to study the basic developmental biology and pathophysiology of these diseases in vitro and to create new opportunities for discovering small molecule therapeutics for their treatment.

Cancer II - Invasion/Metastasis (1423 – 1450)

1423/B642

Understanding RhoC and VEGF-mediated Human Tumor Cell Metastasis using Transparent Zebrafish and High Resolution Intravital Microscopy.

R. Klemke, K. Stoletov, E. Zardouzian; Pathology, UCSD, La Jolla, CA

Understanding the in vivo mechanisms of tumor metastasis has been limited by the inability to image this dynamic, multi-step process in live animals. In vivo imaging is especially challenging for tumor cell intra and extravasation since these metastatic steps occur with low frequency and deep within the tissue. Recently, we have developed a novel zebrafish xenograft model that allows high resolution intravital confocal imaging of the dynamics of cancer progression including microtumor formation, cell invasion, angiogenesis, and vascular penetration (Proc. Natl. Acad. Sci. USA. 2007. 104 (44):17406-11). Using optically transparent zebrafish, we have shown that the human metastatic gene, RhoC, promotes an amoeboid mode of cancer cell invasion characterized by formation of dynamic membrane blebs and protrusions. These dynamic structures serve as specialized invadopodia that penetrate through small vascular openings induced by VEGF secretion leading to increased cell intravasation. This animal model is also a powerful system to visualize the dynamics of tumor cell

extravasation. Human tumor cells injected into the host vasculature lodge in small capillaries, extensively remodel the vessel wall, invade the endothelium, and then extravasate into surrounding tissue. The remodeling vessels are characterized by increased vessel wall thickness, irregular endothelial cell-cell junctions, and prominent endothelial cell membrane ruffling. VEGF secretion by the tumor cells increases local vessel remodeling and tumor cell extravasation which requires VEGFR and src kinase activity. We are currently investigating how known metastatic genes such as RhoC and Twist promote tumor cell extravasation and whether activation of the VEGFR/src/VE-cadherin signaling pathway plays a role in tumor cell vascular invasion. Our results provide a high resolution picture of cancer progression and provide novel insight into the in vivo mechanisms that govern cancer cell metastasis at the tumor cell-vascular interface.

1424/B643

Characterization of a Biological Scaffold as a Novel In Vitro Model for Cancer Cell Invasion.

A. Parekh[1], K. Branch[1], N. S. Ruppender[2], P. D. Boyer[2], S. Guelcher[2], A. Weaver[1,3]; [1]Cancer Biology, Vanderbilt University, Nashville, TN, [2]Chemical & Biomolecular Engineering, Vanderbilt University, Nashville, TN, [3]Pathology, Vanderbilt University, Nashville, TN

Recent experimental studies suggest that the physical and mechanical properties of the tumor microenvironment play a pivotal role in affecting cancer cell phenotype and malignancy. Although current in vitro models based on biological hydrogels such as gelatin and reconstituted basement membrane (BM) have been crucial in studying invasive events, they lack the proper biochemical, physical, and mechanical properties relevant to in vivo BM properties. Therefore, we tested the biological, tissue engineering scaffold urinary bladder matrix (UBM) as a novel in vitro substrate to study cancer cell invasion. This scaffold is composed of well-defined structural and functional proteins that maintain their complex three-dimensional architecture and includes an intact BM. This study was designed to characterize the biophysical and biomechanical properties of the BM derived from UBM (UBM-BM) using swelling experiments and dynamic mechanical analysis. A swelling ratio of 3 indicated a high degree of cross-linking. Stress-strain measurements at low strain rates indicated that the UBM-BM was very rigid with a Young's modulus of approximately 500 MPa. In addition, MCF10A-CA1d breast cancer cells formed invasive, invadopodia-like structures that appeared to penetrate the UBM-BM as observed with immunohistochemistry and fluorescent microscopy. These characteristics reveal that true BM is drastically different than current in vitro models in many aspects relevant to regulating cellular behavior and phenotype and is a suitable tissue analog for elucidating the invasive mechanism.

1425/B644

TβRIII Restores Normal Cytoskeleton Mechanics in Ovarian Cancer Cells.

V. Swaminathan[1], K. Mythreye[2], G. C. Blobe[2], E. OBrien[1], R. Superfine[1]; [1]Physics and Astronomy, UNC, Chapel Hill, NC, [2]Medicine, Duke University, Durham, NC

In cancer, changes in cellular phenotypes are characterized by altered cytoskeletal mechanics as well as disruption of signal transduction pathways controlling proliferation, angiogenesis and apoptosis. Loss of expression of the TGF-b superfamily co-receptor, TbRIII/betaglycan, occurs in a broad spectrum of cancers, including those of the breast, ovary and prostate. Recent studies have shown that restoration of TbRIII to model metastatic populations of ovarian cancer cells reduced migration and restored a more normal cytoskeletal phenotype. We used a magnetic force system (3DFM) to compare the mechanical properties of normal ovarian surface epithelial cells, ovarian cancer cells with TβRIII absent, and ovarian cancer cells with restored TβRIII receptors. Tracking fibronectin-coated magnetic beads during both passive movements and applied forces, we found that the cancer cells without TβRIII were at least 3X more compliant (less stiff) than either normal epithelial or TβRIII-expressing cells. Our results are consistent with potential invasiveness being correlated with cell compliance, show that compliance measurements with the 3DFM could be a useful tool to measure invasiveness in the future, and underscore the role that the TbRIII receptor and downstream signaling pathways may play in the metastatic process.

1426/B645

The Role Played by ADAMTS-1 on Migration and Invasion of Malignant Mammary Gland Cells in 3D Culture.

V. Freitas[1], F. Siviero[1], F. Salles[2], R. Jaeger[1], G. Machado-Santelli[1]; [1]Departament of Cell and Developmental Biology, Institute of Biomedical Sciences, University of Sao Paulo, Sao Paulo, Brazil, [2]Section on Structural Cell Biology, NIDCD, NIH, Bethesda, MD

ADAMTS-1 (a disintegrin and metalloprotease with thrombospondin motifs) is a member of ADAMTS family of metalloproteases. This enzyme is related to pathological processes such as inflammation, cancer development and progression. In spite of ADAMTS-1 biological relevance, the mechanisms underlying its involvement in tumor biology remain elusive. We have been studying the role played by ADAMTS-1 regulating the behavior of a malignant mammary cell line (MDA-MB-231). Immunofluorescence and qPCR showed expression of ADAMTS-1 in MDA-MB-231 cells grown either as monolayers or on Matrigel 3D. To further explore ADAMTS-1 impact on MDA-MB-231 cells we used RNAi to knock down this enzyme. After silencing ADAMTS-1, different aspects of MDA-MB-231 behavior were analyzed. Migration and invasion are important steps in tumor progression. Here we addressed the influence of ADAMTS-1 on three-dimensional migration of MDA-MB-231 cells. Cells had ADAMTS-1 silenced by siRNA. MDA-MB-231 cells transfected with scrambled siRNA served as control. Immunoblot confirmed silencing efficiency. Treated and control cells were grown on Matrigel-Alexa488. To follow migration cells were loaded with Cell Trace Far Red. Growth of cells in fluorescent Matrigel was used to image invasion spots. MDA-MB-231 migratory activity in Matrigel 3D was assessed by multichannel 4D confocal microscopy (UltraView Yokogawa spinning confocal, Perkin Elmer, Waltham, MA, USA), with z-sections taken at different time points. A series of 10 multichannel (Matrigel-Alexa488 and Cell Trace Far Red) confocal sections were collected sequentially for each time point for a total of 30 minutes and three-dimensionally reconstructed by the Volocity software (Improvision, Waltham, MA, USA). Our results showed that control cells spread, migrated and digested Matrigel after 15 min in culture. On the other hand cells with silenced ADAMTS-1 focally digested Matrigel, but failed to spread and migrate even after 30min in culture. Our results suggested that ADAMTS-1 plays a role in three-dimensional migration of MDA-MB-231 cells. Support: FAPESP (2006/54963-0) and CNPq (470779/2007-1).

1427/B646

The Aspartic Proteinase Napsin A Suppresses Tumor Growth in an RGD-Motif Dependent but a Catalytic-Activity Independent

Manner.

T. Ueno[1], M. Toi[1], S. Linder[2]; [1]Breast Surgery, Kyoto University, Kyoto, Japan, [2]Karolinska Institute, Stockholm, Sweden

Napsin A is an aspartic proteinase expressed in the lung and the kidney. In the lung, napsin A is involved in the processing of surfactant protein B although its function in the kidney is unknown. We showed that napsin A mRNA was expressed in normal kidney tissues but detected only in one of 29 renal cell carcinomas, suggesting down-regulation of napsin A during cancer development. To examine the influence of napsin A in tumor growth, HEK293 cells were transfected with napsin A cDNA. Napsin A-expressing HEK293 cells showed an altered phenotype characterized by formation of cyst-like structures in three-dimensional (3D) collagen cultures. Napsin A expressing cells showed a reduced capacity for colony formation in soft agar and tumor growth in SCID mice compared with control cells. Napsin A has some unique structures among aspartic proteinases, including an Arg-Gly-Asp (RGD) motif, which is conserved among species. We examined whether the catalytic activity or the RGD-motif is necessary for tumor suppression. Inactivation of the catalytic site did not influence the cyst-like phenotype in 3D cultures and the ability to suppress tumor formation. In contrast, a mutation in the RGD-motif abolished the ability of napsin A to form cysts in 3D cultures and to inhibit cell growth in soft agar and SCID mice although the mutation did not affect the processing, glycosylation or intracellular localization of napsin A. These data show that napsin A inhibits tumor growth of HEK293 cells by a mechanism dependent on its RGD-motif but independent of its catalytic activity.

1428/B647

Faciogenital dysplasia Protein Fgd1, a Cdc42-specific GEF, Regulates Invadopodia Biogenesis and Extracellular Matrix Degradation and Is Up-Regulated in Prostate and Breast Cancer.

I. Ayala[1], V. Castronovo[2], R. Buccione[1]; [1]Cell Biology and Oncology, Consorzio Mario Negri Sud, S. Maria Imbaro, Italy, [2]Metastases Research Laboratory, Giga-Cancer, University of Liege, Liege, Belgium

Invadopodia are proteolytically active membrane protrusions that extend from the ventral surface of invasive tumoral cells grown on an extracellular matrix (ECM). The core machinery controlling invadopodia biogenesis is regulated by the Rho GTPase Cdc42. We investigated the role of Fgd1, a Cdc42-specific guanosine nucleotide exchange factor (GEF), the loss of which causes the rare inherited human developmental disease faciogenital dysplasia. We found that, in human melanoma A375MM cells, Fgd1 is transiently localized to invadopodia, appearing ahead of invadopodia core components such as cortactin and dispersing before their disassembly. Fgd1 knock-down produced a significant reduction in invadopodia formation and ECM degradation. Wild-type and constitutively active Fgd1 expression increased ECM degradation three-fold compared to control, whereas the constitutively inactive form inhibited degradation 60%. Fgd1 is reported to be specific for Cdc42 but discrepancies sometimes occur between in vitro and in vivo specificities for known GEFs. We thus assayed the levels of active (i.e. GTP-bound) Cdc42 and found them to be 40% lower in Fgd1-depleted cells compared to control. GTP-Rac1, instead, was below detection regardless of Fgd1 expression. Fgd1 also has Cdc42-independent activities in actin remodeling via interaction with cortactin. Our results, however, suggest that Fgd1 acts in ECM degradation only as a Cdc42-specific GEF. This is also supported by the fact that the constitutively active Fgd1 that increases invadopodia formation and ECM degradation lacks the cortactin-binding domains. Finally, we also found that Fgd1 is expressed in human prostate and breast cancer but not in adjacent or control healthy tissues. Expression levels matched lesion aggressiveness and were higher at the invasive edge of the tumor mass. Our findings suggest a central role for Fgd1 in the focal degradation of the ECM in vitro and, for the first time, demonstrate a connection between Fgd1 and cancer progression suggesting that it might play a critical role during tumor progression.

1429/B648

The role of HEF1 in Invadopodia Formation.

R. A. Cline, E. Pugacheva; Cancer Cell Biology, West Virginia University, Morgantown, WV

Human Enhancer of Filamentation 1 (HEF1) is the only member of the Crk-asssociate substrate (Cas) protein family involved in activation of mitotic Aurora A kinase. HEF1 has been found to be over-expressed in tumors and metastases. Over-expression of HEF1 enhances cellular invasion and migration. Initiation of invasion is directly linked to formation of actin based protrusions called invadopodia. The role of HEF1 in invadopodia formation is unknown. We hypothesize that HEF1 enhances invasion through facilitation of invadopodia formation. We have tested this hypothesis on primary cell lines obtained from HEF1 wild type or knockout animals. Primary mouse, human, and tumor cell lines, were assessed on their ability to form invadopodia in the presence of an active form of the Src oncogene (Src 527F), which is shown to stimulate invadopodia formation. Preliminary data shows HEF1 knockout mouse embryonic fibroblasts have a reduced ability to form invadopodia when compared to wild type mouse embryonic fibroblasts when active Src is placed in them. Similar data were obtained when HEF1 was depleted in the highly metastatic breast cancer cell line MDA MB 231. In addition, we tested if Src and cortactin, two major components of invadopodia, were affected by HEF1 over-expression or depletion. We found activation of Src, as well as Serine/Threonine phosphorylation of cortactin, was dependent upon HEF1 expression. Further investigation of HEF1's role in invasion will provide mechanistic explanation of HEF1 induced metastasis and facilitate development of small molecule inhibitors targeting HEF1 in cancer cells.

1430/B649

Human Renal Cancer Cells Express a Novel Membrane-Bound IL-15 That Induces, in Response to the Soluble IL-15Ralpha Chain, Epithelial to Mesenchymal Transition.

B. Azzarone, K. Khawam, P. Eid, J. Giron Michel; U 542, Inserm, Villejuif, France

Epithelial to mesenchymal transition (EMT) is an important change in cell phenotype which allows the escape of epithelial cells from the structural constraints imposed by tissue architecture. Recently evidence has mounted for EMT as the means through which solid tissue epithelial cancers invade and metastasize. EMT is a culmination of several interconnected transduction pathways controlled by a defined set of growth factors, cytokines and downstream transcription factors. EMT plays a central role in renal pathology (chronic allograft rejection and cancer) and IL-15 is a pro-inflammatory factor potentially involved in both pathologies, but not yet correlated to EMT process, herein we investigated the potential relationships between IL-15 and EMT. We show that primary human renal cancer cells (RCC) constitutively express a novel form of membrane bound IL-15 of 27 kDA (tmb-IL-15), that can be triggered by soluble specific ligand, (s-IL-15Rα),

inducing a complex reverse signal necessary and sufficient to induce the EMT. EMT starts with the activation of the MAPkinases ERK1/2, p38 and JNK/SAP, together with the phosphorylation of the Focal Adhesion Kinase (FAK) that are associated with an increased synthesis of αSMA and by the phosphorylation of the myosine light chain. These molecular events contribute to the dynamic reorganization of the cytoskeleton with subsequent formation of αSMA stress fibers, and assembly of vimentin intermediate filaments network. On the other hand, the generation of a mesenchymal/contractile phenotype is associated with the loss of the epithelial markers e-cadherin and ZO-1 that further contribute to the induction of the EMT. In addition, RCC secrete two isoforms of sIL-15Rα that could act as natural ligand of RCC mbIL-15. These signalling functions are however hindered through an unprecedented cytokine-receptor interaction of the mb-IL-15 with IL-15Rα subunits, present on the same cell that tunes in cis its signalling potential competing with low concentrations of the sIL-15Rα. In conclusion, human primary RCC express a mb-IL-15 which displays unusual biochemical and functional properties necessary and sufficient to induce EMT.

1431/B650

Mutations in DLC-1's (Deleted in Liver Cancer 1) Focal Adhesion Targeting Region Attenuate Their Expression and Function.

Y. Shih, Y. Liao, S. Lo; Department of Biochemistry and Molecular Medicine, Center for Tissue Regeneration and Repair, University of California-Davis, Sacramento, CA

Deleted in liver cancer-1 (DLC-1) is a RhoGAP domain containing tumor suppressor that is often down-regulated in various cancer types. Previously, we have demonstrated that DLC-1 is recruited to focal adhesions by binding to the SH2 (Src homology 2) domains of tensins and the focal adhesion localization is critical for DLC-1's tumor suppression activity. To investigate whether mutations in the focal adhesion targeting (FAT) region might occur and attenuate DLC-1's expression, localization, and function, we have first mapped the FAT region to the amino acid residues from 201 to 500, and then sequenced cDNAs and genomic DNAs encoding the FAT region from cancer patients. Several missense and nonsense mutations were detected. All missense mutations were further examined for the potential effect on DLC-1's function. Although these mutations did not appear to affect DLC-1's focal adhesion localization, the activities of suppressing tumor cell growth were impaired in two mutants: T301K and S308I. In consistent with the fact that the RhoGAP activity of DLC-1 is essential for inhibiting tumor cell growth, the RhoGAP activities were significantly reduced in these mutants, suggesting that the FAT region also contains a regulatory element for its C-terminal RhoGAP domain. Our studies have demonstrated that mutations in DLC-1 may lead to loss of function and contribute to tumorigenesis, and have revealed an allosteric regulation site for its RhoGAP activity.

1432/B651

Activations of c-Abl-PKC-delta and Raf1-MEK-ERK Signaling Pathways are Two Critical Events for the Claudin-1-Induced Cellular Invasion.

C. Yoon, M. Kim, J. Byun, S. Lee; Hanyang University, Seoul, South Korea

Claudins are recently identified members of the tetraspanin family of proteins, which are integral to the structure and function of tight junction. Recent studies showed changes in expression of claudins during tumorigenesis. However, a causal relationship between claudin expression and cancer development has not been established. In this study, we show that claudin-1 plays a crucial role in hepatocellular carcinoma (HCC) cell invasion. siRNA targeting of claudin-1 completely inhibited HCC cell invasion. Conversely, overexpression of claudin-1 disturbs cell-cell adhesion, and induced invasive behavior in normal liver cells. We also showed that activations of c-Abl-PKC-delta and Raf1-MEK-ERK signaling pathways are two critical events for the induction of cellular invasion in response to claudin-1 expression. The present observations raise the possibility of exploiting claudin-1 as a potential biomarker for liver cancer progression and might provide new opportunities for therapeutic intervention.

1433/B652

Twist Induced Migration and Invasion of Breast Epithelial Cells is EGF Dependent.

S. Li[1,2], C. A. Glackin[2]; [1]Graduate School of Biological Sciences, City of Hope Beckman Research Institute, Duarte, CA, [2]Molecular Medicine, City of Hope Beckman Research Institute, Duarte, CA

Over the past five years, an estimate of 200,000 patients died of breast cancer in the US. Despite an increase in general survival rate, breast cancer patients with distant metastases continue to live a low survival rate compared to those with carcinoma in situ. However, the cause of metastasis is still unknown. Twist, an evolutionarily conserved transcription factor that regulates mesoderm formation, was recently found to over-express in many epithelial carcinomas and correlate with metastatic potential. In order to understand how Twist mediates metastasis, we stably over-expressed Twist (MCF10ATw, 10ATw) in a human breast epithelial cell line (MCF10A, 10A) to study its function. Twist over-expression resulted in distinctive morphological changes in 10A cells, converting them from stereotypic epithelial to a disseminated structure with membrane protrusions. Migration and invasion assays confirmed that 10ATw cells were highly motile and invasive. When we further examined the mechanism of the changes, we found that the morphology that 10ATw cells displayed was dependent on the presence of EGF, which was used to supplement the culture medium. When EGF was withdrawn from the medium, 10ATw cells lost their motile/invasive morphology and acquired an 10A-like phenotype. Moreover, no detectable migration was seen when EGF was absent. To dissect the molecular basis of this phenomenon, we performed quantitative PCR and found a 2.2 fold increase of EGFR in 10ATw cells compared to 10A cells. In addition, inhibition of MEK kinase with U0126 led to an epithelial-like phenotype, similar to that of 10ATw cells cultured in the absence of EGF. However, no changes were observed with the PI3K inhibitor, LY294002. These results suggest that EGFR/MEK/ERK signaling pathway mediates the Twist induced changes in 10ATw cells. By demonstrating the correlation of Twist and EGFR, we show that Twist can not only change the physical connection between cells but alter cellular responses to extracellular environment. When placed in a microenvironment coupled with activated EGFR ligands, Twist over-expressing cells can become more motile and invasive, which may eventually disseminate from the original tumor bed and metastasize.

1434/B653

Down-Regulation Of Bnip3 via Autophagy Promotes the Migration and Invasion of Hepatocellular Carcinoma Cells by Increasing

the Expression Level of Twist1.

C. Park, E. Kim, K. Choi; Dept. of Life Science, POSTECH, Pohang, South Korea

Objective : Autophagy is an evolutionarily-conserved homeostatic process that can remove cellular proteins and cytoplasmic organelles in response to stress or starvation. Autophagy is known to play an important role in cancer. However, it is still unclear whether autophagy inhibits tumorigenesis or rescues cancer cells under stress conditions. Bcl-2/adenovirus E1B 19 kDa interacting protein-3 (Bnip3) is a pro-death molecule of the Bcl-2 family that is expressed in response to hypoxia. Recently, Bnip3 was shown to inhibit the progression of metastasis . Therefore, we investigated if autophagy can promote the metastasis of cancer cells by regulation of Bnip3. Method : We induced autophagy via amino acid starvation in SH-J1, a liver cancer cell line derived from a patient. After the induction of autophagy, we assessed the change of cell motility through the migration and invasion assay, and the expression level of Bnip3 by western blot analysis. Also, we knock-downed Bnip3 with siRNA and observed the difference of cell migration and invasion as well as the expression level of Twist1. Result : We found that starvation-induced autophagy promoted the migration and invasion of SH-J1 while the Bnip3 level was highly down-regulated. The knock-down of Bnip3 was found to increase the rate of migration and invasion of SH-J1. Twist1, which was known to be essential for the metastasis of cancer cells, was up-regulated in the abscence of Bnip3. Starvation-induced autophagy promoted the migration and invasion of SH-J1 which could express high level of Bnip3. Conclusion : Our results suggest that Bnip3 be down-regulated by autophagy, which could increase the expression level of Twist1 and enhance the migration and invasion of SH-J1. Deprivation of oxygen (hypoxia) and deficiency of nutrient (autophagy) seem to be synergistically involved in the selection of metastatic cancer cells.

1435/B654

TRIM29 is a Potential Tumor Suppressor in Estrogen Receptor-Positive Breast Cancer.

J. Liu, P. Bernard; Huntsman Cancer Institute, Salt Lake City, UT

Tripartite motif-containing 29 (TRIM29) is a member of the TRIM protein family. Gene expression microarray analysis shows differential expression of TRIM29 in breast cancer with higher expression in estrogen receptor (ER)-negative than ER-positive tumors. Kaplan-Meier survival analysis indicates that ER-positive patients with high TRIM29 expression have better overall survival and relapse-free survival than those of patients with low TRIM29 expression, suggesting that TRIM29 is a disease-specific prognosis marker in ER-positive breast tumors. In order to investigate the role of TRIM29 in breast cancer initiation and progression, we transfected the normal-like breast cell line MCF10A, which has high TRIM29 expression, with lentiviral vectors containing TRIM29 shRNA to knockdown endogenous TRIM29. Stably transfected cell lines were then selected for cell-based assays, three-dimensional (3D) Matrigel cell culture and gene expression microarray analysis. Results from cell-based assays demonstrate that the inhibition of TRIM29 expression resulted in increased cell proliferation and induction of anchorage-independent growth. In addition, metastasis characteristics of MCF10A cells, including cell migration and cell invasion to basement membrane were also significantly enhanced upon TRIM29 knockdown. In 3D basement membrane culture, silencing of TRIM29 in MCF10A cells induced pre-neoplastic changes during mammary acinar morphogenesis, including loss of polarity, increased proliferation and diminished apoptosis. Transfection of a TRIM29 construct refractory to shRNA silencing into TRIM29-knockdown cells led to the nearly full rescue of phenotypic changes in 3D culture. Moreover, findings from gene expression microarray of the transfected cell lines correlate to results from in vitro functional studies. Specifically, TRIM29 inhibition is accompanied by down-regulation of genes in p53 tumor suppressor pathway and up-regulation of genes that are associated with increased cell proliferation. Genes implicated in transformation and metastasis were also up-regulated in TRIM29-knocked down cells. Taken together, these data show that TRIM29 may act as a tumor suppressor in ER-positive breast tumors.

1436/B655

Nischarin over Expression Inhibits Metastasis by Restoring Epithelial Markers in Metastatic Breast Cancer Cells.

S. Baranwal[1], A. Walch[1], J. Lakins[2], V. Weaver[2], S. Alahari[1]; [1]Biochemistry and Molecular Biology, LSUHSC, New Orleans, LA, [2]Surgery, University of California, San Francisco, CA

Nischarin, originally discovered as a cytoplasmic binding partner of alpha 5 integrin, is an important protein that regulates cell adhesion and cell migration. Previously we showed that Nischarin inhibits tumor cell migration and invasion of breast cancer cells by specifically affecting p21 activated kinase (PAK) signaling. Most recently we determined that Nischarin blocks LIMK (a downstream effector of PAK) activation. Our previous data indicate that Nischarin levels are lower in highly invasive breast cancer cells and tissues. In addition, xenograft studies suggested that Nischarin overexpression significantly reduces the tumor volume suggesting that Nischarin may function as a breast tumor suppressor. In the current study, our nude mouse experiments showed that human metastatic breast cancer cells co-expressing Nischarin with luciferase (Nisch+ MDA MB231 luciferase) demonstrated a significant delay in the onset of tumor metastasis as compared to parental controls (cont-MDA MB231 luciferase). To understand the molecular mechanism by which Nischarin could inhibit metastasis, we examined the effect of Nischarin on epithelial-mesenchymal transition (EMT). Interestingly, overexpression of Nischarin in MDA-MB231 cells restored expression of epithelial marker proteins including E-cadherin and γ-catenin, and reduced the expression of mesenchymal proteins such as Fibronectin and Vimentin. Nischarin also down-regulated levels of Snail1, a key transcription factor that modulates EMT. These data suggest that Nischarin inhibits metastasis by restoring the expression of epithelial markers that results in the reversal of the EMT process, and establish a mechanistic connection between Snail, EMT and metastasis. Supported by grants from NIH 5RO1CA115706, Susan Komen BCTR0600278, LA board of regents LEQSF-RD-A-14 to S.K.A, and NIH grant 7RO1CA078731-07 and DOD W81XWH-05-1-330 to V.M.W.

1437/B656

The O-GlcNAc Modification on Transcriptional Repressor Snail Regulates the Stability of Snail.

S. Park[1], W. Yang[1], S. Ji[1], J. Yook[2], J. Cho[1]; [1]Biology, Yonsei University, Seoul, South Korea, [2]Oral Pathology, Yonsei University, Seoul, South Korea

Epithelial-mesenchymal transition (EMT) is a program of development of biological cells characterized by loss of cell adhesion and increased cell mobility. EMT is required in many developmental processes, such as gastrulation and neural crest migration. In the

pathological aspect, deregulation of EMT can induce tumor progression in cancer cells. Snail is transcriptional repressor of E-cadherin, which regulates cell adhesion in epithelial cells, and the repression of E-cadherin is thought to play a major role in the abnormal manifestation of EMT. O-GlcNAc modification is a post-translational modification, which is occurred in nucleus and cytosol. Many proteins, including transcription factors, cytoskeletal proteins and various enzymes, are known to be O-GlcNAcylated. Here we firstly report that snail is an O-GlcNAcylated protein and O-GlcNAc modification on snail increases the stability of snail. In the hyperglycemic conditions, snail protein was accumulated, but mRNA level did not increase. Snail is known to be down-regulated by phosphorylation which is a signal for ubiquitin-proteasome protein degradation pathway. When O-GlcNAcylation of snail was increased, ubiquitination on snail was decreased by inhibition of phosphorylation. Furthermore, we identified that Ser112 was O-GlcNAcylated site by mass spectrometry and mutagenesis study. In the case of substitution mutant of snail(S112A), there was no accumulating effect by the hyperglycemic conditions or the treatments of O-GlcNAcase inhibitors.

1438/B657

SCAI is a Suppressor of Cancer Cell Invasion by Regulating MAL and β1 Integrin Gene Expression.

D. Brandt[1], C. Baarlink[1], T. Kitzing[1], P. Nollau[2], R. Grosse[1]; [1]Institute of Pharmacology, University of Heidelberg, Heidelberg, Germany, [2]Department of Clinical Chemistry, University of Hamburg, Hamburg, Germany

Reprogramming of gene expression controls complex processes such as cell migration through transcriptional cofactors. Here we describe SCAI (Suppressor of Cancer Cell Invasion), a novel protein that regulates invasive cell migration. SCAI acts on the RhoA-Dia1 signal transduction pathway by accumulating in the nucleus where it binds and inhibits the myocardin-related transcription factor MAL by forming a complex with SRF. Genome-wide target gene analysis in human cancer cells reveals that SCAI regulates β1 integrin. Reduced SCAI levels correlate with increased invasive cell migration and SCAI is downregulated in human tumors. Functional studies on the β1 integrin gene suggest that SCAI is a novel transcriptional cofactor that regulates gene expression downstream of the formin Dia1 to dictate changes in cancer cell invasion.

1439/B658

Effects of p120 Ablation in Normal and Transformed Mammary Epithelium.

S. Kurley[1], M. A. Davis[1], W. J. Muller[2], A. Reynolds[1]; [1]Cancer Biology, Vanderbilt University, Nashville, TN, [2]Biochemistry and Medicine, McGill University, Montreal, QC, Canada

p120-catenin (p120) modulates epithelial cell-cell adhesion by controlling the stability of E-cadherin, an important suppressor of tumor progression and metastasis. Areas of p120 loss are observed in most carcinoma types, including ~10% of ductal breast carcinomas, but the significance of this observation is unclear. Here, we have used conditional p120 knockout mice by methods of Cre-loxP technology to examine the in vivo effects of p120 ablation in normal and transformed mammary epithelium. Inducing p120 deletion at the onset of mammary development (MMTV-Cre; p120F/F) delayed ductal outgrowth initially as seen by whole mount analysis. However, p120 null cells were rapidly lost and replaced by p120 positive cells that behaved normally. Thus, p120 appears to be essential for normal mammary ductal development. To examine p120 loss in the context of tumor progression, we crossed our p120 knockout mice into the MMTV-Polyoma Middle T (PyMT) mouse model of breast cancer described previously by Dr. W. Muller (resulting in MMTV-Cre; MMTV-PyMT; p120F/F animals). Interestingly, the PyMT oncogene rescued p120 null mammary epithelial cells resulting in mixed PyMT tumors containing both p120 positive and p120 negative regions. p120 ablation induced a pseudo-papillary phenotype, reticular deposition of collagen and infiltrated fibrovascular stroma. E-cadherin was almost completely lost in PyMT tumors lacking p120. Indeed, the histology of these tumors phenocopied invasive lobular breast cancer, know to be correlated with loss of E-cadherin expression. Lung metastases in these animals were both p120 positive and negative, suggesting that p120 is not essential for metastasis. Currently, we are evaluating additional cases to determine whether metastasis is accelerated or increased by p120 ablation in this mouse model.

1440/B659

Arginyltransferase as a Potential Regulator of Tumorigenesis.

R. R. Rai[1], J. Wang[1], M. Shtutman[2], F. Zhang[1], S. Kurosaka[1]; [1]Animal Biology, University of Pennsylvania, Philadelphia, PA, [2]Ordway Research Institute, New York, NY

Arginylation is a poorly understood posttranslational protein modification mediated by arginyltransferase (Ate1) that is essential for embryogenesis. Higher mammals, including mice, express four Ate1 isoforms with differential activity, tissue specificity, and intracellular localization. Our past work demonstrated that Ate1 regulates multiple proteins in vivo, including a prominent subset of cancer-related targets, however the role of Ate1 in regulating the tumorigenic properties of cells was never addressed before. To study the role of Ate1 in cancer, we studied tumorigenic properties of fibroblasts derived from Ate1 knockout mice and the correlation between Ate1 levels and cell tumorigenicity. We found that immortalized Ate1 knockout cells grew faster and reached much higher densities in culture than the wild type control. This growth correlated with prominent changes in morphology of ATE1 knockout cells, which grew on top of each other after reaching the monolayer and exhibited apparent defects in contact inhibition. When grown in Matrigel, Ate1 knockout cells, unlike wild type, formed colonies and exhibited invasive behavior characteristic for malignant cells. Moreover, these cells could form tumors when injected into immunodeficient mice. Studies of the role of deletion or overexpression of individual Ate1isoforms in wild type and Ate1-knockout cells on their morphology, growth rate, and tumor-forming behavior, as well as the correlation of general Ate1 levels with tumorigenesis in vivo are in progress.

1441/B660

The Poor Prognosis Indicator Podocalxyin Alters Epithelial Architecture and Cellular Adhesion in Breast and Ovarian Carcinoma Cells without Inducing an EMT.

M. Graves[1], J. A. Cipollone[1], M. Koebel[2], J. Nielsen[3, 4], B. Gilks[2], K. M. McNagny[3], C. D. Roskelley[1]; [1]Cellular and Physiological Sciences, The University of British Columbia, Vancouver, BC, Canada, [2]The Genetic Pathology Evaluation Center, Vancouver General Hospital and

BC Cancer Agency, Vancouver, BC, Canada, [3]The Biomedical Research Center, The University of British Columbia, Vancouver, BC, Canada, [4]Deeley Research Center, British Columbia Cancer Agency, Victoria, BC, Canada

Podocalyxin is an apically targeted sialomucin normally expressed at the surface of luminal mammary epithelial cells and ovarian surface epithelial cells, the likely target tissues of the majority of breast and ovarian carcinomas respectively. Using tissue microarray technology, we have found that overexpression of podocalyxin in invasive human breast tumors is an independent predictor of tumor progression and poor outcome (Somasiri et.al. (2004) Can.Res. 64; 5068-5073). Podocalyxin is also expressed in the majority of ovarian carcinomas, but is most prevalent in high-grade serous tumors, which form the most common and lethal ovarian carcinoma subtype. In podocalyxin-positive serous tumors, subcellular targeting to the free surface was an indicator of poor outcome (p=0.03). When podocalyxin was force expressed into non-metastatic breast and ovarian carcinoma cells it was preferentially targeted to the free surface membrane, which altered the apical membrane architecture and recruited the multi-functional adaptor molecule NHERF-1 and the ERM-family member ezrin into actin rich protrusions. Ectopic podocalyxin at the free-surface membrane also caused a decrease in homotypic cell-cell aggregation and inhibited tumor cell adhesion to ECM, at least in part, by masking beta 1 integrins and altering their localization. Additionally, podocalyxin disrupted the growth and architecture of 3D tumor spheroids, but without significantly altering cell-cell junction formation. Thus, podocalyxin may be functionally important in tumor sub-types where metastatic progression is initiated in the absence of an overt epithelial to mesenchymal transition as occurs in some ductal breast carcinomas where polarity disruption is prominent, and in most high-grade serous ovarian tumors where the detachment of tumor cell clusters from the ovarian surface is a pre-requisite for transperitoneal spread.

1442/B661

Loss of HEF1/NEDD9 Limits Breast Cancer Progression.

E. Izumchenko[1,2], O. V. Plotnikova[1], M. Singh[1], S. Seo[3], M. Kurakawa[3], M. Wolfson[2], E. Golemis[1]; [1]Basic Science, Fox Chase Cancer Center, Philadelphia, PA, [2]Microbiology and Immunology, Ben-Gurion University, Beer-Sheva, Israel, [3]University of Tokyo, Tokyo, Japan

The HEF1 scaffolding protein regulates pathways involving TGF-β, Src, FAK, and Aurora-A that drive cell transformation. Upregulation of HEF1 characterizes metastatic spread of different forms of cancer; and overexpression of HEF1 promotes metastasis. It is currently unknown whether HEF1 plays a role in early stages of cancer to predispose individuals for metastatic diseases but this is plausible based on its interactions. We hypothesized that lack of HEF1 may limit tumor metastasis, or other early stages of the transformation process. To test this hypothesis, we crossed HEF1-/- mice to MMTV-PyVT transgenic mice in which breast-specific expression of the PyVT oncogene causes rapid onset primary breast adenocarcinomas that subsequently metastasize to the lung. HEF1-/- status decreased the total tumor burden and the number of affected breast pads and increased the latency of primary breast tumors. Analysis of pre-malignant lesions revealed an early effect of HEF1-/- status in slowing tumor growth. Although HEF1-/- status induces some late immune cell defects, we have excluded differences in immune response to tumor or tumor vascularization as explaining the difference in tumor appearance. To define the mechanistic basis for the decreased malignancy of HEF1-/- tumors, we used cell lines derived from the primary breast tumors, and tissue samples collected from experimental animals. Intriguingly, primary mammary tumor lysates from HEF-/- animals show a significant reduction in Src, FAK and MAPK phosphorylation when compared to the lysates obtained from the tumors of HEF+/+ animals. The degree to which these pathways fail to activate in individual animals correlates with latency of tumor onset. These data indicate that inability to upregulate HEF1 significantly reduces tumor malignancy.

1443/B662

A Role of mDia1 in Src-induced Malignant Transformation and Invasion.

M. Tanji, T. Ishizaki, S. Narumiya; Pharmacology, Kyoto University, Kyoto, Japan

c-Src, one of the most thoroughly investigated proto-oncogenes, has a significant role in development of human cancer. The mechanism of Src activation has been intensively investigated, and previous studies indicate that actin rearrangement by Rho GTPases recruits Src to adhesion complexes and then to the peripheral membrane. However, the contribution of this pathway to Src-mediated malignant transformation remains to be tested. mDia1 is a Rho effector and a formin molecule catalyzing formation of long, straight actin filaments. Here we used mouse embryonic fibroblasts (MEF) from mDia1-deficient mice, temperature-sensitive v-Src mutant and examined the role of mDia1 in v-Src-induced malignant transformation. MEFs were prepared from wild type and mDia1-/- littermate mice and immortalized by retroviral transfection of SV40 antigens. They were then transfected by ts-v-Src (NY72). A population of the wild type MEF (ts-v-Src) (WT-NY72) cells and that of the knockout MEF (ts-v-Src) (KO-NY72) cells were established. While the two populations exhibited similar levels of expression of v-Src protein, KO-NY72 cells did not show morphological transformation such as disruption of stress fibers and formation of podosomes at permissive temperature as observed in WT-NY72 cells. Consistently, WT-NY72 cells failed to show morphological transformation upon depletion of mDia1 by RNAi. Furthermore, KO-NY72 cells exhibited significantly impaired foci formation and impaired colony formation in soft agar compared to WT-NY72 cells. When injected subcutaneously into nude mice, KO-NY72 cells showed impaired tumor formation with less invasiveness. When the two populations of cells were subjected to spreading assay or to a shift to the permissive temperature, WT-NY72 cells exhibited translocation of v-Src from the perinuclear region to the periphery while such translocation was impaired in KO-NY72 cells. These results suggest that the Rho-mDia pathway contribute to expression of Src-mediated malignant transformation by recruiting Src molecules to cell periphery.

1444/B663

R-Spondin2/Int7 and Wnt-1 Stimulate Invasiveness and Tumorigenicity of Mammary Epithelial Cells.

M. Klauzinska[1], A. Raafat[1], L. Strizzi[2], B. Baljinnyam[3], Y. Endo[3], J. S. Rubin[3], R. Callahan[1]; [1]Oncogenetics Section, Mammary Biology and Tumorigenesis Laboratory, National Cancer Institute, Bethesda, MD, [2]Children's Memorial Research Center, Robert H. Lurie Comprehensive Cancer Center, Northwestern University Feinberg School of Medicine, Chicago, IL, [3]Laboratory of Cellular and Molecular Biology, National Cancer Institute, Bethesda, MD

Mouse R-spondin2 (Rspo2) is a member of the R-spondin protein family, which has been recently described as a set of secreted molecules with ligand-type activities partially overlapping Wnt ligands. Moreover, Rspo2 was identified as a novel common integration site for the

mouse mammary tumor virus (MMTV). Interestingly, the tumors with an MMTV insertion at Rspo2/Int7 site were also found to have additional viral insertions flanking a member of either the Wnt and/or FGF gene family suggesting that there may be collaboration between R-spondin2/Int7 and Wnt/Fgf genes during mammary tumorigenesis. Here, we showed that overexpression of Rspo2 in two mouse epithelial cell lines, HC11 and C57MG, alters their basic features and activates the β-catenin pathway. Furthermore, Rspo2 is able to synergize with Wnt1 in the canonical β-catenin signalling pathway. However, it reverses Wnt-1- induced morphological transformation of mouse epithelial cells. Our in vivo studies showed that cells expressing Wnt-1 and Rspo2 independently can develop into tumors in nude mice and that these genes when co-expressed can synergize to confer an invasive capability in 3D Matrigel assays. In contrast, silencing of the key protein of the canonical Wnt pathway, β-catenin, did not have any effect on invasive capability of C57MG-Wnt1Rspo2 cells, suggesting that this aspect of Rspo2-mediated transformation of mouse mammary epithelial cells is β-catenin independent.

1445/B664

ANXA7 Functions as a Tumor Suppressor in Human Glioblastomas.

A. K. Yadav[1], J. J. Renfrow[1], J. P. Chandler[1], H. Vogel[2], B. I. Sikic[2], G. R. Harsh[2], M. Bredel[1,2]; [1]Northwestern University, Chicago, IL, [2]Stanford University, Stanford, CA

Background: Glioblastoma multiforme is one of the most devastating human tumors. The molecular biology of glioblastoma is complex, involving multiple genetic alterations. Hemizygous loss of chromosome 10q is one of the most frequent chromosomal aberrations in glioblastomas. The alterations of several tumor suppressor gene residents on 10q might contribute to a 10q loss-of-function phenotype in glioblastoma. Annexin VII (*ANXA7*) is a candidate tumor suppressor on 10q21.1-q21.2. We hypothesize that *ANXA7* is a haploinsufficiency gene and that hemizygous loss of *ANXA7* is an important event in gliomagenesis. **Methods:** We profiled *ANXA7* at genetic and transcript levels in a cohort of 45 human gliomas using microarray methodology. The *ANXA7* gene was silenced in several glioblastoma cell lines and normal human astrocytes via retroviral transfection of short hairpin (sh) RNAs targeting ANXA7 mRNA, mimicking a hemizygous gene loss. Retroviral transfection was also used to overexpress *ANXA7* in the glioblastoma cell lines. The biological effect of *ANXA7* knockdown or overexpression was studied using various phenotypic assays. **Results:** We found a significant effect of *ANXA7* gene dosage on transcription in the glioma specimens. We found that shRNA-based knockdown of ANXA7 in glioblastoma cells results in spontaneous foci formation in culture and increased clonogenicity in soft agar, which is associated with increased and sustained activation of the MAPK and PI3K-AKT signaling pathways. In turn, overexpression of *ANXA7* attenuates tumorigenic potential, decreases tumor cell viability and migratory potential, and induces tumor cell senescence. ANXA7 knockdown in normal human astrocytes induces a transformed phenotype as evidenced in matrigel culture and spheroid formation in cell culture. **Conclusions:** ANXA7 loss-of-function in human glioblastomas and normal human astrocytes promotes malignant transformation. *ANXA7* might thus have a bona fide tumor suppressor role in glioblastoma, wherein it functions to constrain tumorigenic potential and tumor cell proliferation.

1446/B665

A Focal Adhesion Molecule, Cten, Is a Novel Modifier for APC.

Y. Liao, N. Chen, S. Lo; Department of Biochemistry and Molecular Medicine, Center for Tissue Regeneration and Repair, University of California, Davis, Sacramento, CA

Cten (C-terminal tensin like), a tensin family member, is a Src homology 2 (SH2) and phosphotyrosine binding (PTB) domain-containing focal adhesion molecule. Cten regulates the formation of actin cytoskeleton and focal adhesions, cell migration, apoptosis, and is involved in tumor progression. Its expression is readily detected in the prostate and placenta, but extremely low, if any, in other tissues, such as the colon. Nonetheless, we recently found that cten mRNA and protein levels were consistently up-regulated in human colon cancer. Since $Apc^{Min/+}$ mice are commonly used as a mouse colon cancer model, we examined cten expression levels in $Apc^{Min/+}$ mice. In agreement with human colon cancer results, we found cten mRNA was consistently overexpressed in all tumor samples. To examine a potential role of the cten signaling in tumorigenesis, we generated $Apc^{Min/+}$ mice in cten-deficient background and analyzed sex- and age-matched cohorts. We found that lack of cten expression significantly reduced the progression and the size of adenoma formation. To further link cten to the APC-Wnt signaling pathway, cten expression in colon cancer cell lines was examined. We detected that cten proteins was only over-expressed in cell lines expressing APC mutants but not in cells expressing wild type APC together with constitutively active β-catenin mutants. Furthermore, β-catenin knockdown has no effect on cten expression. Our studies have demonstrated that cten is a new β-catenin-independent modifier of APC in tumor development.

1447/B666

STAT3-deficient Fibroblast Cells Demonstrate Uncontrolled Proliferation, Elevated PDGF Receptor and phospho-Erk Levels.

L. G. Rodriguez[1], A. M. Zovanyi[1], P. Castillo Martinez[2], S. J. Lockett[1]; [1]Advanced Technology Program, SAIC-Frederick, Inc, Frederick, MD, [2]Department of Biology, University of Puerto Rico, Rio Piedras Campus, NIH Summer Intern Program, Hatillo, PR

Signal Transducer and Activator of Transcription 3 (STAT3) is a member of the STAT family of proteins. STAT3 has been shown to play a positive role in many cancers, as activated STAT3 has been found present in many clinical tumor samples. In these studies, we demonstrate that STAT3-deficient mouse embryonic fibroblast (ST3) cells continue to proliferate under normal culture conditions, unlike normal mouse embryonic fibroblast (MEF) cells that cease proliferation due to a confluent saturation density. Additionally, we show, utilizing a cell based ELISA, that the expression of PDGF receptor and phospho-Erk are elevated in ST3 cells relative to normal MEF cells. Finally, preliminary data shows that reintroduction of STAT3 into ST3 cells restored the control of proliferation due to the confluent saturation density exhibited by normal cells. These results suggest an anti-proliferative role for STAT3 in fibroblast cells, resulting from cell-cell contact.

1448/B667

Fatty Acids Associated with Increased Breast Cancer Risk Alter Apical Polarity in Breast Epithelium.

L. S. Chaboub, J. M. Cardenas-Mora, M. M. Bethany, S. Lelièvre, K. Metcalf; Purdue University, West Lafayette, IN

Intake of high ratios of ω-6/ω-3 polyunsaturated fatty acids (PUFAs) has been linked to breast cancer occurrence. Our laboratory has identified disruption of apical polarity, as measured by tight junction proteins distribution, as a potentially important event in breast cancer development. Notably, studies using three-dimensional cultures of non-neoplastic breast epithelial cells that have differentiated into tissue-like mammary acini show that loss of apical polarity primes cells to enter the cell cycle. Our goal is to investigate the impact of ω-3 and ω-6 PUFAs on apical polarity. We show that ω-3 PUFAs eicosapentaenoic acid and alpha-linolenic acid do not affect acini differentiation; however, ω-6 PUFAs arachidonic acid (AA) and linoleic acid, as well as high, western diet type, ratios of ω-6/ω-3 PUFAs induce a loss of apical polarity in acini after a four-day treatment, as shown by the altered distribution of tight junction marker ZO-1 revealed by fluorescence immunostaining. To identify the molecular pathway by which ω-6 PUFAs alter apical polarity, we treated acini with prostaglandin E2 (PGE2), a product of AA metabolism that participates in inflammatory reactions and is overexpressed by breast cancer cells. PGE2 treatment resulted in an increased number of mammary acini without correct apical polarity compared to controls; in contrast, anti-inflammatory PGE3 did not perturb polarity. Increased activation of E-Prostanoid receptors (EP2) and (EP4) has been linked to cancer cell proliferation. AH6809 and AH23848, which inhibit EP2 and EP4, respectively, were used to treat acini in combination with AA or PGE2. Results suggest that inhibition of EP2 and EP4 receptors protects from the loss of apical polarity upon AA or PGE2 treatment. Acini treated with AA were also incubated with LY294002 to inhibit phosphoinositide-3 kinase (PI3K), an enzyme activated as a result of EP4 stimulation. Data indicate that LY294002 prevents AA-induced tight junction alteration. These results suggest that PUFAs might increase the likelihood for mammary epithelial cells to become cancerous via the EP4/PI3K and/or EP2 inflammatory pathways. We are currently investigating how the EP4/Pi3K pathway may destabilize apical polarity.

1449/B668

ZBRK1 Acts as a Tumor Suppressor and Inactivated through the E2F1/Rb/CtIP/CtBP Complex.

C. Liao[1], J. Wang[2]; [1]Basic Medical Science, Tainan, Taiwan, [2]Ins. of Biosignal Transduction, NCKU, Tainan, Taiwan

ZBRK1 (zinc finger and BRCA1-interacting protein with a KRAB domain-1) is a KRAB-ZNF that contains a highly conserved KRAB domain at the NH2 terminus, eight consecutive C2H2 zinc finger motifs at the COOH terminus, and a CTRD domain. We recently found that the E2F-site mutation of ZBRK1 promoter resulted in the enhancement of reporter activity. Furthermore, the E2F1/Rb complex is identified to involve in this negative regulation through the recruitment of CtIP and CtBP, the recently clarified transcriptional suppressors, by the DNA binding assay and reporter assays. On the other hand, the growth rate of constitutively expressing ZBRK1 cells was markedly reduced compare with the control cells in foci assay and soft agar assay. The silencing ZBRK1 is observed in cervical cancer and hepatocellular carcinoma. Furthermore, the blockage of tumor formation suggests that ZBRK1 has a potential to prevent tumor growth and its downregulation may take an advantage for tumorigenesis.

1450/B669

Groucho Family Protein Amino-terminal Enhancer of Split AES is Induced by the AML1-ETO Fusion Protein and Important for Self-Renewal in Acute Myeloid Leukemia.

M. Knop[1], B. Steffen[2,1], C. Stocking-Harbers[3], B. Koerner[1], S. Doths[1], E. Bulk[1], N. Baeumer[1], B. Schulte[4], M. Stehling[5], W. Berdel[1], H. Serve[2,1], C. Mueller-Tidow[1]; [1]Molecular Hematology & Oncology - Medical Clinic A, University of Muenster, Muenster, Germany, [2]Medical Clinic II, University of Frankfurt, Frankfurt, Germany, [3]Heinrich-Pette Institute - Molecular Pathology, University of Hamburg-Eppendorf, Hamburg, Germany, [4]Gerhard-Domagk Institute of Pathology, University of Muenster, Muenster, Germany, [5]Molecular Biomedicine, Max Planck Institute, Muenster, Germany

Recently, the Wnt-signaling pathway has been identified as an important player in hematopoiesis and pathogenesis of acute leukemias. We found several genes associated with Wnt-signaling as target genes of chimeric fusion proteins like AML1 ETO, PML-RARα and PLZF-RARα in Acute Myeloid Leukemia (AML). One identified gene regulated by fusion proteins is AES (amino-terminal enhancer of split) belonging to the TLE/groucho (grg) family proteins. AES lacks the repression domain and inhibits repression of other groucho/TLE. In this study we analyzed the influence of AES in differentiation and self-renewal of hematopoietic progenitors. AES knockdown by RNAi in AML1-ETO expressing hematopoietic cells resulted in significantly reduced cloning and plating efficiency. In the absence of AES 32D cells showed reduced colony growth and increased apoptosis. In murine bone marrow AES expression leads to enhanced formation of immature colonies and serial replating capacity of these primary cells. AES expression was increased in AML patients versus normal bone marrow samples. Therefore AES serves as a marker for self-renewal in hematopoietic progenitors. In this analysis we present new findings concerning relevant target genes in transcriptional regulation of AML development.

Cell Biology of the Immune System I (1451 – 1470)

1451/B670

Polarization of Dendritic Cells at the Immune Synapse Facilitates Local Delivery of T Cell Priming Cytokines.

J. Pulecio[1], J. Petrovic[1], P. Massimi[2], F. Prete[1], L. Banks[2], F. Benvenuti[1]; [1]Molecular Immunology, International Center for Genetic Engineering and Biotechnology, Trieste, Italy, [2]Tumor Virology, International Center for Genetic Engineering and Biotechnology, Trieste, Italy

Dendritic cells (DCs) engage in immune synapse (IS) with T cells during the initiation of adaptive immunity. This cell-cell interaction facilitates molecular communication between membrane proteins like TCR /MHC peptide complexes and co-stimulatory molecules, and ensures spatially restricted delivery of soluble mediators. Formation of IS in T cells is accompanied by asymmetric distribution of cytoskeletal and signalling proteins that reorient towards the antigen-presenting cell. The molecular events that link TCR signaling to T cell polarity have been characterized quite extensively. In contrast, little is known about polarization events occurring in DCs during IS formation. Here we have investigated cell polarity in DCs forming conjugates with naïve CD8+ T cells. We found that Toll-like receptor (TLR) stimulated DCs rapidly polarize the microtubule-organizing center (MTOC) toward the T cell in an antigen- dose dependent manner.

In DCs deficient for the Wiskott-Aldrich syndrome protein, a key regulator of cytoskeletal dynamics, MTOC reorientation is dramatically reduced. To further define polarity in DCs we studied proteins of the scribble polarity network that were recently shown to play a role in T cell polarity. We found that the Disc Large protein 1 (Dlg1) is highly expressed in DCs and is recruited at the interface with the T cell in close proximity to the MTOC. To address the functional role of polarization in DCs we analyzed the intracellular distribution of IL-12, a key cytokine produced by DCs upon TLR stimulation. Intracellular stores of IL-12 were detected in a perinuclear Golgi region closely associated to the MTOC. Upon synapse formation the MTOC-associated intracellular pool of IL-12 was translocated at the cell-cell contact in close proximity to the T cell membrane. Quantification of the volume of the cytokine containing vescicles inversely correlates with the distance from the synapse region, suggesting that the cytokine is secreted in a polarized fashion toward the T cell. In summary this study illustrates a new mechanism in DCs to efficiently transport cytokines produced upon TLR stimulation to the DC-T cell junction thus enhancing local delivery of T cell priming mediators.

1452/B671

Separation Forces in T Cell-APC Interaction Studied by Atomic Force Microscopy.

B. H. Hosseini[1,2], I. Louban[1,2], D. Djandji[3], J. P. Spatz[1,2], G. J. Hämmerling[3]; [1]New Materials and Biosystems, Max Planck Institute for Metals Research, Stuttgart, Germany, [2]Biophysical Chemistry, University of Heidelberg, Heidelberg, Germany, [3]Molecular Immunology, German Cancer Research Center, Heidelberg, Germany

During adaptive immune responses peptides from foreign antigens and pathogens are processed and displayed on Antigen Presenting Cells (APCs) within the cleft of major histocompatibility complexes (pMHC). T cells patrolling lymphoid tissues scan APCs and establish cell-cell contacts when their antigen-specific T Cell Receptors (TCRs) recognize cognate foreign peptide on the APC's surface. At the contact zone of these cell conjugates spatially organized molecular clusters are established and collectively referred to as the immune synapse (IS). The formation of the IS involves the coordinated translocation of many surface molecules, including two major protein complexes: TCR and its ligand pMHC, as well as the integrin lymphocyte function-associated antigen-1 (LFA-1) and its counterpart: intercellular adhesion molecule 1 (ICAM-1). Little is known about the biophysics of these cell conjugates and it is debated whether forces play a vital role in this process. Here, we report the first measurement of separation forces between T cells and APCs employing Atomic Force Microcopy (AFM). We studied several time points and used APCs without peptide as control. Our setup consists of a JPK NanoWizard II mounted on an upright Zeiss microscope enclosed by environmental conditions. Cantilevers were functionalized with antibodies to ensure firm adhesion of T cells. Hybridomal B cells acting as APCs were plated on poly-l-lysine (PLL) coated glass cover slips. Using fluorescent dye techniques immune synapses formed between the two cells were visualized. We analyze the kinetics of T cells interacting with APCs presenting peptide by their MHC (pulsed), and APCs carrying no foreign peptide (unpulsed) for contact times of 0-60 minutes. Unlike pulsed cells, unpulsed APCs did not show the formation of immune synapses. Forces are comparable for short time points and start to deviate after 15min. Unpulsed cells are not able to establish strong cell-cell contacts whereas APCs carrying cognate peptide show a 10-fold increase of adhesion forces. These findings are consistent with current models involving an inside-out signaling mechanism where the entire translocation machinery is triggered by peptides present on the MHC molecules.

1453/B672

Long Distance Intercellular Communication via Natural Killer Cell Membrane Nanotubes.

A. Chauveau[1,2], E. Vivier[2], D. Davis[1]; [1]Cell and Molecular Biology, Imperial College London, London, United Kingdom, [2]Centre d'Immunologie Marseille Luminy (CIML), Marseille, France

Membrane nanotubes are membranous tethers that can connect cells over long-distances and are commonly detected between immune cells. Recent data has revealed considerable heterogeneity in their structure, process of formation and functional properties, in part dependent on the cell types involved (Nat Rev Mol Cell Biol, 9, 431, 2008). Here, we show that when human Natural Killer (NK) cells make contact either with other NK cells or with target cells and subsequently depart, they commonly remain connected by a membrane nanotube. Such nanotubes connect 3-10% of cells in culture and have an average length of 20.5 ± 1.3 µm, but can be >100 µm. The frequency of membrane nanotube formation can be at least in part controlled by the number of receptor/ligand interactions between cells. For example, the frequency of nanotube formation between cells can be increased 3-fold when target cells are transfected to express an additional NK cell ligand. As recently found for nanotubes between T cells (Nat Cell Biol, 10, 211, 2008), nanotubes connecting NK cells and target cells are distinct from open-ended membranous tethers and contain a dynamic junction. By live cell imaging, we observed an accumulation of the signaling adaptor protein DAP10 that associates with the activating receptor NKG2D, as well as an accumulation of the NKG2D ligand MICA, at such junctions. Quantitatively, there was a striking 5 to 20-fold increase of these proteins at the nanotube junction compared to elsewhere along the nanotube. This was not due to an accumulation of membrane as confirmed by simultaneous imaging of a membrane dye. Such protein clusters within synapses can form either before or after the nanotube formation and were commonly sustained for periods of at least 30 minutes. In addition, a large aggregation of tyrosine-phosphorylated proteins accumulated with MICA and DAP10 at the nanotube junction. Taken together, these data provide the first evidence for a role of membrane nanotubes in facilitating long-distance intercellular signaling between NK cells and target cells and suggests that nanotube junctions may represent functional submicron-scale synapses.

1454/B673

A Quantitative Proteomic Approach Reveals Regulators of Antigen Receptor-Induced Membrane-Cytoskeletal Dynamics in B Lymphocytes.

N. Gupta[1,2], B. Wollscheid[3], B. Scheer[2], J. Watts[3], R. Aebersold[3], A. DeFranco[2]; [1]Immunology, Cleveland Clinic, Cleveland, OH, [2]Microbiology & Immunology, University of California San Francisco, San Francisco, CA, [3]Institute for Systems Biology, Seattle, WA

Recent biochemical evidence indicates that an early event in signal transduction by the B cell antigen receptor (BCR) is its translocation to glycosphingolipid- and cholesterol-rich membrane subdomains in the plasma membrane known as lipid rafts. Our live cell imaging analysis of antigen-triggered B cells revealed recruitment of key proximal signaling molecules to these domains as well as aggregation of lipid rafts into increasingly large domains until they coalesce into micron-sized caps at one pole of the cell. Lipid raft coalescence has also been

reported to occur after crosslinking of a variety of other signaling receptors and is thought to potentiate cellular activation. To identify positive and negative regulators of lipid raft dynamics during BCR signaling, we have employed a mass spectrometry-based systems biology approach. We have used solid-phase isotope-coded affinity tags (ICAT) to carry out a quantitative analysis of the proteome of lipid rafts from B cells before and after stimulation with anti-IgM. Our data revealed a change in the relative abundance of several proteins, including the membrane-cytoskeleton crosslinker ezrin, and the molecular motor Myh9. Biochemical and microscopic validation of the proteomics data demonstrated that ezrin was rapidly dephosphorylated upon BCR crosslinking, causing it to adopt a closed conformation that detaches from actin as well as from lipid rafts. Moreover, expression of constitutively active ezrin chimeras that linked the plasma membrane to the actin cytoskeleton interfered with BCR-induced lipid raft coalescence. Therefore, our data support the hypothesis that dissociation of ezrin from lipid rafts is a critical trigger that releases the plasma membrane lipid rafts from the underlying cortical actin cytoskeleton and allows activation-induced lipid raft coalescence to proceed. The ability of ezrin to regulate membrane-cytoskeletal dynamics in B cells may confer on it the potential to shape B cell responses to antigen.

1455/B674

Negative Regulation of T Cell Mediated Immune Responses via PEA-15 Modulation of ERK Signaling.

S. Pastorino, H. Renganathan, J. E. Gawecka, A. Modic, J. W. Ramos; Natural Products and Cancer Biology, University of Hawaii at Manoa, Honolulu, HI

The Mitogen-Activated Protein Kinase (MAPK) Extracellular-signal-Regulated Kinases (ERKs) can phosphorylate a diverse set of substrates and can thereby control transcription, cell proliferation, survival, and adhesion. In T lymphocytes, ERK modulates proliferation, partly by inducing IL-2 transcription and by competing with a SHP-1 dependent negative feedback pathway. The Death Effector Domain-containing scaffold protein PEA-15 is an ERK-binding protein that can restrict ERK1/2 to the cytoplasm and thereby prevent ERK activation of nuclear targets. Here we show that PEA-15 controls ERK signaling in T cells upon TCR stimulation. PEA-15 deficiency resulted in increased TCR-induced nuclear translocation of ERK, and increased activation of IL-2 transcription and production. Moreover, PEA-15 deficient mice showed increased proliferation of T cells upon TCR stimulation and increased antigen-specific antibody production in vivo. Our results indicate that PEA-15 negatively regulates TCR signaling and T cell-mediated immune responses by modulation of the ERK pathway. This work provides new insight into the control of T cell activation and provides the first indication that PEA-15 is an important regulator of T cell homeostasis.

1456/B675

Eupatilin, a Single Compound Purified from Artemisa Plants, Inhibits T Cell Activation through Blocking of Calcium Influx.

Y. Kim[1], T. Oh[2], S. Choi[3], C. Jun[1]; [1]Life Science Department, Gwangju Institute of Science and Technology, Gwangju, South Korea, [2]Dong-A Pharmaceutical Co. Ltd., Research Institute, Yongin, South Korea, [3]Wonkwang University School of Medicine, Digestive Disease Research Institute, Iksan, South Korea

Controlling T cell immunity is one of the attractive subjects in the field that immunosuppression is necessarily required. Here we investigated whether eupatilin inhibits T cell activation. Treatment of Jurkat T cells with eupatilin dramatically reduced TCR-mediated or PMA/A23187-mediated IL-2 production. Eupatilin neither affected the accumulation of CD3 or LFA-1 molecules in the immunological synapse nor inhibited the T and B cell conjugation and TCR internalization. Although eupatilin alone induced apoptotic cell death in Jurkat T cells, interestingly, rather it inhibited activation-induced cell death, suggesting that apoptosis is not a corresponding mechanism of eupatilin-mediated inhibition of T cell activation. Otherwise, we found that eupatilin specifically inhibits calcium flux in resting T cells or in cells treated with various stimuli including CD3, PHA, or A23187. Subsequently, eupatilin dramatically inhibited NF-κB and NF-AT activation in Jurkat T cells. Collectively, the present results demonstrate that eupatilin inhibits T cell activation by blocking intracellular calcium flux and the down-stream signaling pathways.

1457/B676

Estrogen Upregulates the Interferon-α Pathway in T Cells of Women with Systemic Lupus Erythematosus.

E. Walters[1], V. Rider[1], N. I. Abdou[2], C. Greenwell[2], S. Svojanovsky[3], P. Smith[3]; [1]Biology, Pittsburg State University, Pittsburg, KS, [2]Center for Rheumatic Diseases, St. Luke's Hospital, Kansas City, MO, [3]Department of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, KS

Systemic lupus erythematosus (SLE) is an autoimmune disease that occurs primarily in women (10:1 compared to men). We used gene profiling to identify signaling pathways that are differentially regulated in SLE T cells by estrogen. Blood samples were collected from SLE patients (n= 6) with active disease [systemic lupus erythematosus disease activity index (SLEDAI, > 5) and from control females (n = 6) age matched to the patients. Purified T cells were cultured without and with estradiol 17-β (10-7 M) for 18 h and activated with PMA and ionomycin for an additional 4 h. T cell RNA was isolated, converted into biotinylated cRNA and hybridized to high density Affymetrix human GeneChips. The chips were scanned and analyzed using the MicroArray Suite (Affeymetrix). The Ingenuity Pathways Analysis library was used to identify pathways that were differentially regulated by estrogen in SLE T cells. The interferon α pathway increased significantly in SLE T cells in response to estrogen (p-value = 4.68*10^-3, Fischer's exact test). To confirm DRIP150 as an estrogen dependent target in SLE T cell interferon α signaling, RNA was purified from T cells cultured and activated as for microarray analysis. RNA (4 μg) was reverse transcribed and the resulting cDNAs were amplified by real time PCR. A comparative gene expression method, utilizing a DRIP150 Taq Man (Applied Biosystems) assay and glyceraldehyde 3-phosphate dehydrogenase as an internal control, is beginning to confirm the microarray findings. The mean fold change in DRIP150 expression in response to estrogen for the activated SLE T cells (n = 3) was 2.12, compared with a mean fold change of 1.12 for the activated control T cells (n = 6), and 1.20 for the activated RA T cells (n = 2, disease controls). While previous research revealed that the interferon α pathway is stimulated in SLE, the importance of estrogen in this regulation has not been reported. These data suggest that upregulation of interferon α signaling in SLE T cells is modulated by estrogen and suggest that estrogen stimulation of this pathway may contribute to the development of gender biased autoimmunity. Funded in part by NIH AI49272 and RR-16475 from the INBRE Program of the NCRR.

1458/B677

Saikosaponin-d (Ssd) Inhibits Mouse T Lymphocyte Activation and Its Pro-inflammatory Cytokines Production through Suppression of IκBα Kinase and Akt Activation.

K. Wong, T. Li, H. Zhou, L. Liu; School of Chinese Medicine, Hong Kong Baptist University, Hong Kong, China

Saikosaponin-d (Ssd) is a triterpene saponin derived from the medicinal plant, *Bupleurum falcatum* L. (Umbelliferae). Previous findings showed that Ssd exhibits a variety of pharmacological and immunomodulatory activities including anti-inflammatory, anti-bacterial, anti-viral and anti-cancer effects. In the current study, we performed the transcription factor assay, kinase assay, western blotting, flow cytometry analysis and ELISA to investigate the effect of Ssd on activated mouse T lymphocytes through the NF-κB signaling pathway, cytokine secretion, and IL-2 receptor and CD71 antigen expression. Our results demonstrated that Ssd inhibited PMA, PMA/Ionomycin and Con A-induced T cell activation in vitro. The inhibitory effect of Ssd on PMA-induced T cell activation was associated with down-regulation of NF-κB signaling through suppression of IKK and Akt activity. The cell surface markers like IL-2 receptor (CD25) and transferrin receptor (CD71) were also down-regulated together with decreased production of several pro-inflammatory cytokines of IL-2, IL-6, TNF-α and IFN-γ. In conclusion, our study elucidate the underlying mechanisms of Ssd with its potent immunosuppressive effect and suggest its potential to be used to treat T cell-mediated autoimmune conditions.

1459/B678

Immunogenecity of Modified Alkane Polymers is Mediated through TLR1/2 Receptor Activation.

C. C. Clement[1], R. Maitra[1], G. M. Crisi[2], N. Cobelli[3], L. Santambrogio[1]; [1]Pathology, Albert Einstein College of Medicine, Bronx, NY, [2]Pathology, Baystate Medical Center, Springfield, MA, [3]Orthopedic Surgery, Montefiore Medical Center, Bronx, NY

With the advancement of biomedical technology, artificial materials have been developed to replace diseased, damaged or nonfunctional body parts. Among such materials, ultra high molecular weight alkane or modified alkyl polymers have been extensively used in heart valves, stents, pacemakers, ear implants, as well as total joint replacement devices. Although much research has been undertaken to design the most non-reactive biologically inert polyethylene (PE) derivatives, strong inflammatory responses followed by rejection and failure of the implant have been noted. The molecular mechanisms underlying these clinically important events are very poorly understood. This research aimed to determine the molecular mechanisms for the PE recognition by the immune system. Our histological evaluation of periprosthetic implant associated tissue, retrieved from patients undergoing hip replacement surgery due to PE induced osteolysis, indicated chronic inflammatory infiltrates. Immunophenotyping demonstrated that these infiltrates consisted predominantly of CD68+ macrophages, with few T and B cell infiltrates. Histiocytes and foreign body giant cells engulfing birefringent PE particles, generated by wear and tear of the polymeric implant, were also observed. Ultrastructural analysis revealed the presence of micron and sub-micron PE particles in the periprosthetic inflamed tissue, similar to the one derived from chemically synthesized carbonyl modified alkane polymers. Purification of the alkane polymers from the site of inflammation revealed extensive "in vivo" oxidation as detected by fourier transformed infra-red spectroscopy. In addition, we report the novel observation that oxidized alkane polymers induced activation of TLR1/2 pathway as determined by ligand dependent changes in intrinsic tyrosine fluorescence intensity and NF-kB luciferase gene assays. Oxidized polymers were very effective in activating dendritic cells and inducing secretion of pro-inflammatory cytokines. Molecular docking of the oxidized alkanes designated ligand specificity and polymeric conformations fitting into the TLR1/2 binding grooves. This is the first report of a synthetic polymer activating immune responses through TLR binding.

1460/B679

GCN2 Kinase Pathway in Differential Response of Human Cells to Indoleamine 2, 3-Dioxygenase (IDO)-Induced Low Tryptophan Environment: T cells vs Skin Cells.

F. Forouzandeh[1], R. Jalili[1], V. Duronio[2], S. Boyce[3,4], A. Ghahary[1]; [1]Surgery, University of British Columbia, Vancouver, BC, Canada, [2]Medicine, University of British Columbia, Vancouver, BC, Canada, [3]Surgery, University of Cincinnati, Cincinnati, OH, [4]Research, Shriners Burns Hospital, Cincinnati, OH

Objective: Indoleamine 2, 3- dioxygenase (IDO) is a heme-containing rate-limiting enzyme, catalyzes conversion of tryptophan to kynurenine. We have previously demonstrated that IDO expressed by dermal fibroblasts generates a tryptophan deficient environment in which immune cell lines such as THP-1 and Jurkat cells, but not skin cells, undergo apoptosis. However, it is not clear whether the primary immune cells such as CD4+ and CD8+ T cells which are responsible for graft rejection would do so under similar experimental condition. Further, the mechanism by which the primary skin cells such as fibroblasts and keratinocytes are resistant to this culture environment is not known. Methods: To address these issues, primary immune cells were co-cultured with IDO expressing fibroblasts and their rate of proliferation and General Control Nonderepressing-2 (GCN2) kinase pathway activity in response to allogeneic antigens were evaluated by cell proliferation assay and western blot analysis, respectively. Results: The result showed that co-culturing IDO expressing primary human fibroblasts with CD4+ and CD8+ cells significantly suppressed their immune reaction to allogeneic pieces of either epidermis or full skin relative to those co-cultured with non-IDO expressing fibroblasts. This effect was restored by using an IDO inhibitor, 1-methyl-D-tryptophan (1-MT). However, under similar experimental condition, primary fibroblasts were resistant to IDO induced low tryptophan environment. In addition, the results showed a significant activation of apoptotic pathway as analyzed by caspase-3 induction as well as the expression of CHOP, a downstream effector of GCN2 kinase pathway in both T cell subsets, but not in skin cells. Conclusion: The lack of caspases-3 and GCN-2 pathway activation in primary fibroblasts and keratinocytes in response to IDO induced low tryptophan environment differentiates them from CD4+ and CD8+ cells which are very sensitive to the same experimental environment. Therefore, our group is working to make use of the differential effect of IDO expression on primary skin cells vs T cells to develop non-rejectable skin substitute.

1461/B680

Ras Isoforms Differentially Regulate CD40 Signaling In Macrophages.

S. Pahari, B. Saha; National Centre for Cell Science, Pune, India

CD154 - CD40 interactions in macrophages induces counteractive cytokines depending on the strength of signaling; however signaling intermediates remain unknown. Weak CD40 signals induce extracellular stress related kinase-1/2 (ERK-1/2)-dependent interleukin-10 (IL-10) production, stronger signals induce p38 mitogen-activated protein kinase (p38MAPK)-dependent IL-12 production, and intermediate CD40 signals induce both ERK1/2 dependent IL-10 and p38MAPK dependent IL-12 production. As Ras GTPases have been considered an important switch in various signaling events leading to regulation of diverse cellular functions, we started investigating its role in CD40 signaling in macrophages. Stimulation of Manumycin A (a potent Ras inhibitor) treated macrophages with intermediate dose of anti-CD40 suppresses ERK1/2 and p38MAPK activation, suggesting a role of Ras GTPase in CD40 signaling. We also checked the Ras GTPase activation with different doses of anti-CD40 stimulation. Ras GTPases have three isoforms namely H-Ras, K-Ras and N-Ras. Despite their great structural similarity, evidences suggest their functional disparity. Stimulation of H-Ras and K-Ras siRNA transfected murine macrophage cell line with intermediate dose of anti-CD40 resulted in inhibition of p38MAPK activation, whereas in N-Ras siRNA transfected cells, ERK1/2 activation is inhibited. Dominant negative mutant studies and activation studies of H-Ras, K-Ras and N-Ras revealed that N-Ras mediated CD40 signaling is distinct from H-Ras and K-Ras mediated CD40 signaling. Immunoprecipitation and siRNA studies suggested that different dose of CD40 ligation results in differential association of Ras isoforms with their effector molecules Raf-1 and PI3Kinase. Leishmania major, a protozoan parasite skews CD40 signaling in macrophages towards ERK1/2 dependent IL-10 production and inhibits anti-parasitic IL-12 production. We observed significant changes in the expression of Ras isoforms and CD40- induced activation of Ras isoforms in Leishmania-infected and uninfected macrophages. Together, these findings demonstrate that Ras isoforms differentially regulate CD40 signaling in macrophages and are targeted by Leishmania to manipulate CD40 signaling.

1462/B681

IL-17 Producing γδ+ T cells Protected NOD Mice from Type I Diabetes in a Mechanism Involved of TGF-beta.

G. Han[1], R. Wang[1], G. Chen[1], J. Wang[1], R. Xu[1], L. Wang[1], J. Feng[1], X. Li[3], R. Guo[2], B. Shen[1], Y. Li[1]; [1]Department of Molecular Immunology, Institute of Basic Medical Sciences, Beijing, China, [2]Department of Pathology, University of Michigan Medical School, Michigan, MI, [3]Laboratory of Cellular and Molecular Immunology, Henan University, Kaifeng, China

The role of Th17 cells and their cytokine IL-17 in autoimmune diabetes remain largely unclear. Here we found that, although diabetic T cells produced higher IL-17 than normal ones, neutralization of IL-17 could not protect diabetes transferred by diabetic T cells in NOD-scid mice. Diabetic T cells expanded by rIL-23 showed enhanced capability of IL-17 production but did not induce increased diabetes incidence after transfer. Different from the production of IFN-g which peaks on insulitis onset ages of NOD mice then gradually decreased thereafter, the production of IL-17 keeps unchanged at different age's levels. As we found that IL-17 was mainly expressed by γδ+T rather than CD4+T cell in NOD mice, and purified gd+T cells are dominated by IL-17+ expressing cells and secreted 10-fold higher IL-17 than total T cells in response to rIL-23 stimulation, to test the roles of IL-17 producing γδT cells in autoimmune diabetes, purified γδ+T cells were injected into NOD mice, or were co-transferred with diabetic T cell into NOD-scid mice. Unexpectedly, IL-17 producing γδT cells protected NOD mice from diabetes by up-regulating TGF-beta production. Our data here indicated that IL-17 is not the critical pro-inflammatory cytokine in the pathogenesis of autoimmune diabetes in NOD mice. γδT cells, although produced higher level IL-17, protect NOD mice from diabetes in a mechanism involved of TGF-beta.

1463/B682

Fibrinogen Binding to ICAM-1 Promotes EGFR-Dependent Mucin and IL-8 Production via Inhibition of an Epithelial Surface Signaling Cascade.

S. Kim[1,2], J. L. Koff[1,2], J. A. Nadel[1,2]; [1]Cardiovascular Research Institute, Univ. of California San Francisco, San Francisco, CA, [2]Dept. of Medicine, Univ. of California San Francisco, San Francisco, CA

Mucins and IL-8 are produced and secreted in the airways as part of innate immune responses to inhaled invaders. Here we show that binding of fibrinogen (an ICAM-1 ligand) to ICAM-1 promotes epidermal growth factor receptor (EGFR)-dependent mucin and IL-8 production in human airway (NCI-H292) epithelial cells: An ICAM-1 neutralizing antibody and an ICAM-1 (8-22) peptide that binds fibrinogen decreased transforming growth factor (TGF)-alpha-induced mucin and IL-8 production dose-dependently. Exogenous fibrinogen and a fibrinogen (117-133) peptide that binds ICAM-1 rescued mucin and IL-8 production in cells treated with the ICAM-1 (8-22) peptide. The ICAM-1 (8-22) peptide increased TGF-alpha-induced EGFR phosphotyrosine and phospho-ERK1,2. These increases were prevented by exogenous fibrinogen and by fibrinogen (117-133) peptide. In addition, selective inhibitors of phospholipase C (PLC), protein kinase C (PKC)-alpha/beta, and metalloproteases prevented the increases in TGF-alpha-induced EGFR phosphotyrosine and phospho-ERK1,2 in cells treated with the ICAM-1 (8-22) peptide. Taken together, these results suggest that fibrinogen binding to ICAM-1 promotes TGF-alpha-induced mucin and IL-8 production via inhibition of PLC- and PKC-alpha/beta-dependent activation of a metalloprotease and inhibition of subsequent metalloprotease-dependent EGFR reactivation.

1464/B683

Endotoxin-Mediated Hetero-Tolerance of Interleukin-8 Production by the Ribotoxic Stress in Intestine-407 Cells.

S. Park, Y. Moon; Department of Microbiology and Immunology, Pusan National University School of Medicine, Busan, South Korea

Pre-exposure to the commensal endotoxins can induce inflammatory tolerance and maintain the homeostasis without excessive immune responses. We recently reported that ribotoxin deoxynivalenol elicited the pro-inflammatory response as the mucosal insults in human epithelial cells. Taking the knowledge into consideration, we tested the hypothesis that endotoxin pre-exposure can attenuate ribotoxin-induced epithelial interleukin-8 (IL-8) production via a tolerance mechanism. Pre-exposure to endotoxin repressed IL-8 release and its gene expression. However, inflammatory tolerance was not mediated by the attenuated NF-kappaB activation which has been generally recognized as the major mediator of LPS-mediated toll like receptor (TLR) signaling pathway. Instead, pre-exposure to endotoxin was observed to trigger the delayed induction of peroxisome-proliferator activated receptor gamma (PPAR-gamma) which contributed to the diminished IL-8 production in the human epithelial cells. Moreover, endogenous PPAR-gamma agonist suppressed toxicant-mediated interleukin-8

production and IL-8 mRNA stability. Taken together, endotoxin induced hypo-production of pro-inflammatory cytokine IL-8 in the human epithelial cells, which was associated with the delayed activation of PPAR-gamma expression by pre-existing endotoxin.

1465/B684

Potential Roles for the Protein Agrin in Regulating Aspects of the Innate Immune Response.

M. Mencel[1], C. Jacobson[1,2]; [1]Biology, University of Waterloo, Waterloo, ON, Canada, [2]Mechanical & Mechatronics Engineering, University of Waterloo, Waterloo, ON, Canada

Agrin is a heparan sulfate proteoglycan best known for its role in acetylcholine receptor (AChR) clustering during the formation of the post-synaptic apparatus of the neuromuscular junction. Interestingly, both agrin and AChRs are broadly expressed in a variety of tissues outside of the nervous system. For instance, AChRs are present on non-excitatory cells of the innate immune system, such as macrophages and neutrophils; cells that have been implicated in regulating inflammation in response to the presence of infection via a neuro-immune axis. This has been termed the cholinergic anti-inflammatory pathway wherein the vagus nerve releases acetylcholine (ACh). ACh activates the α7 nicotinic AChRs (α7-nAChRs), inhibiting the synthesis and production of pro-inflammatory cytokines, such as tumor necrosis factor (TNF-) α and interleukin (IL-) 1β, the key mediators of inflammation. We find that agrin increases α7-nAChR expression in two murine cell lines: the macrophage cell line RAW264.7 and the neutrophil cell line MPRO. In addition, agrin appears to aggregate surface α7-nAChRs in these cell lines. These findings suggest a role for agrin in modifying an inflammatory response by the innate immune system by regulating the efficacy of vagus derived ACh.

1466/B685

Repression of STAT1 and Caspase-11 by Hypoxia-Inducible Factor-1α.

H. Cho, H. Park; Life Science, University of Seoul, Seoul, South Korea

Macrophage infiltrates into tissue in response to inflammatory stimuli. Recently, O2 concentration in normal tissue is estimated about 2-9 %. Therefore hypoxic effect should be considered to study the function of macrophage in inflammation process. LPS activates macrophages to express cytokines and caspases such as IL-1β and caspase-11 by activating transactivators NF-κB and STAT1. Caspase-11 is involved in cleavage of pro-IL-1β. We found that hypoxia attenuates the LPS-induced expression of caspase-11 and IL-1β by repressing STAT1 expression in murine macrophage RAW 264.7 cells. We found that HIF-1α and its target STRA13/DEC1, transcription repressor are involved in hypoxic repression of STAT1 and caspase-11. Overexpression of STRA13 or HIF-1α in normoxic NIH-3T3 fibroblast cells reduced expression of STAT1 and caspase-11, whereas siRNAs targeting STRA13 or HIF-1α increased expression of STAT1 and caspase-11. Our results showed that HIF-1α and its target Stra13/DEC1 involve the hypoxic repression of STAT1, thereby decreasing expression of caspase-11 and IL-1β. These findings emphasize that HIF-1α is a good target for developing cell-protective drug. [This work was supported by a grant (R200706192003) from KOSEF. H-J C is supported by BK21 program]. Ref). Sitkovsky, M., and Lukashev, D. (2005). Regulation of immune cells by local-tissue oxygen tension: HIF1 alpha and adenosine receptors. Nat Rev Immunol 5, 712-721.

1467/B686

Transduced PEP-1-Cyclophilin A (CypA) Efficiently Suppresses LPS-induced Expression of Proinflammatory cytokines and COX-2 in Macrophage Raw 264.7 Cells.

M. Lee, D. Kim, S. Kim, S. Lee, Y. Lee, M. Jeong, H. Jeong, H. Kang, M. Shin, S. Jang, K. Lee, J. Park, W. Eum, S. Choi; Biomedical Science and Research Institute for Bioscience and Biotechnology, Hallym university, Chuncheon, South Korea

Modulation of proinflammatory cytokines and COX-2 production is considered a promising approach to therapy of diseases such as inflammatory disorders. The incidence and severity of anti-inflammatory response is steadily increasing at an alarming rate. Cyclophilins are originally identified as cellular binding proteins for the immunosuppressive drug cyclosporine A (CsA). In the present study, we investigated the suppressive effects of PEP-1-CypA fusion protein on lipopolysaccharide (LPS)-induced cytokines and COX-2 production in macrophage Raw 264.7 cells. The expressed and purified PEP-1-CypA fusion proteins transduced efficiently into macrophage Raw 264.7 cells in a dose- and time-dependent manner when added exogenously in culture media. Once inside the cells, the transduced PEP-1-CypA proteins stable for 24 h. Transduced PEP-1-CyPA fusion protein efficiently suppressed the expression of proinflammatory cytokines, COX-2 and the activities of NF-kB, MAPK in LPS-stimulated macrophage Raw 264.7 cells. Our results demonstrate that transduced PEP-1-CypA fusion protein significantly suppresses LPS-induced inflammatory reactions. Thus, we suggest that PEP-1-CypA fusion protein can be potential candidate for protein therapeutic drug in anti-inflammatory response.

1468/B687

Carbon Monoxide Blocks Lipopolysaccharide (LPS)-Induced Gene Expression by Interfering with Proximal NF-κB Signal Transduction in Human Monocytes.

M. Chhikara, S. Wang, S. J. Kern, R. L. Danner; Clinical Center/Critical Care Medicine Department, National Institutes of Health, Bethesda, MD

Carbon monoxide (CO) is an endogenous messenger generated by heme oxygenase from the cleavage of heme. CO reduces vascular tone, inhibits platelet aggregation, promotes vascular remodeling, dampens inflammation and modulates apoptosis. Unlike nitric oxide, CO only weakly activates soluble guanylate cyclase and for many functions, the proximal targets of CO signaling are unknown. Here, microarrays (Affymetrix 133plus2) were employed to globally characterize CO (250 ppm) suppression of early (1h) LPS-induced inflammation in human THP-1 cells. Ninety-one transcripts were induced by LPS and suppressed by CO (FDR $\leq$ 0.2% and $\geq$ 25% fold change; Genomatix). Of these early-immediate, CO-suppressed transcripts, 16% (15/91) were known transcription factors and most others were cytokines, chemokines and immune response genes. In both THP-1 cells and elutriated human monocytes, real-time RT-PCR was used to validate a substantial subset of the LPS-induced, CO-suppressed transcripts including IL-1β, TNFα, IL-8, ICAM-1, COX-2, CCL20, CXCL-2, PTX3, TNFAIP3, PDE4B, EGR3 and EGR4. CO was also confirmed to down-regulate IL-1β and IL-8 protein production. *In silico* analysis revealed that many CO-suppressed genes had NF-κB binding sites in their proximal promoters. CO was found to suppress LPS-induced phosphorylation and

degradation of IκB-α, as well as the activation and DNA binding of NF-κB in human monocytes. In addition to its effects on NF-κB signaling, CO also caused a very early, transitory delay in LPS-induced JNK activation, suggesting a block at a level above NF-κB inducing kinase (NIK). Thus, CO interferes with LPS-induced signal transduction proximal to NF-κB activation and thereby broadly suppresses gene expression. Therapeutic strategies based on CO itself or utilizing the underlying mechanism of its anti-inflammatory effects may be beneficial in treating pulmonary and vascular inflammation.

1469/B688

Ikarisoside A Inhibits Inducible Nitric Oxide Synthase in Lipopolysaccharide-Stimulated RAW 264.7 Cells via p38 Kinase and Nuclear Factor-Kb Signaling.

Y. Soh, H. J. Choi, Y. R. Park, M. Nepal; School of Dentistry, Dept of Pharmacology, Chonbuk National University, Jeonju, South Korea

Objective: This study examined the anti-inflammatory properties of Ikarisoside A, isolated from Epimedium koreanum (Berberidaceae), in lipopolysaccharide (LPS)-stimulated macrophages. Methods: The murine macrophage cell line (RAW 264.7) was purchased from the American Type Culture Collection (Manasas, VA, USA) and grown in Dulbecco's Modified Eagle's Medium supplemented with 10% heat-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 U/ml). The cells were grown in a humidified atmosphere containing 5% CO2 at 37<o>C. Bone marrow-derived macrophages (BMMs) were isolated from the tibia and femora of 6-week-old ICR mice. Results: Ikarisoside A inhibited the expression of LPS-stimulated inducible nitric oxide synthase (iNOS) and the production of nitric oxide (NO) in LPS-stimulated RAW 264.7 cells and BMMs in a concentration-dependent manner. Ikarisoside A also reduced the release of proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β(IL-1β). Furthermore, Ikarisoside A inhibited p38 kinase and nuclear factor-κB (NF-κB), which are signals involved in NO production. NO production was inhibited when the cells were treated with LPS and either SB 203580 or Bay 11-7082. Conclusion:These results suggest that Ikarisoside A inhibits the production of NO by inhibiting the activity of p38 MAPK and NF-κB. Moreover, Ikarisoside A can be used as a potential anti-inflammatory agent.

1470/B689

Auranofin Up-regulates Heme Oxygenase-1 Expression through Nrf2 Activation via Rac1/NADPH/iNOS Signal Pathway.

N. Kim, M. Oh, S. Park, I. Kim; Natural Sciences, the Catholic University of Korea, Seoul, South Korea

Auranofin, gold(I)-containing antirheumatic drug, has an antiinflammatory function. The pharmacologic effect of auranofin is, in part, dependent on heme oxygenase-1 (HO-1). In the present report, molecular mechanisms for the auranofin-induced HO-1 expression were studied using THP-1 monocytic cells. In THP-1 cells, auranofin increased gene expression of HO-1, corresponded with an increment of nuclear factor-E2-related factor 2 (Nrf2). Auranofin enhanced Nrf2 protein level in a dose- and time-dependent manner but did not affect mRNA level, suggesting that auranofin increases Nrf2 protein stability. Cycloheximide-blocking experiment showed that half life of Nrf2 protein was extended by auranofin from 90 min to more than 180 min. We also found Rac1/NADPH oxidase activation and iNOS induction/NO production in auranofin-treated cells, and the inhibitor of Rac1 (NSC23766) blocked iNOS induction as well as Nrf2 activation and HO-1 expression. L-NAME and AG, inhibitors of iNOS, also intercept the auranofin-induced Nrf2 activation and HO-1 expression. The phosphorylations of MAP kinases were augmented by auranofin treatment, and inhibitors of MAP kinases were partially inhibited the Nrf2 activation and HO-1 expression stimulated by auranofin. To investigate whether the inhibitory effect of auranofin on NF-κB activation caused by the action of the HO-1, we made HO-1-knockdown cells using HO-1-specific siRNA. However, TNF-α-induced IκB degradation was still blocked by auranofin in the HO-1-knockdown cells. These findings indicate that auranofin promotes HO-1 expression through Nrf2 activation which is mediated by Rac1/NADPH/iNOS signaling and MAP kinases activation. However, the auranofin-induced HO-1 may not be involved in the mechanism of NF-κB inactivation in THP-1 cells.

Metabolic Diseases I (1471-1486)

1471/B690

CHC22 Clathrin Heavy Chain Isoform Functions in GLUT4 Transport in Human Insulin Responsive Tissues.

S. Vassilopoulos[1], C. Esk[1], S. Hoshino[1], C. Chen[1], B. Funke[2], W. E. Wright[3], R. Kucherlapati[2], F. M. Brodsky[1]; [1]Biopharmaceutical sciences, UCSF, San Francisco, CA, [2]Partners Healthcare Center for Genetics and Genomics, Harvard Medical School, Boston, MA, [3]Department of Cell Biology, University of Texas Southwestern, Dallas, TX

There are two isoforms of clathrin heavy chain expressed in humans. The CHC17 isoform is ubiquitously expressed and coats vesicles involved in receptor-mediated endocytosis and organelle biogenesis. The CHC22 isoform is primarily expressed in skeletal muscle, to a lesser extent in cardiac muscle, and we have now established that it is also expressed in adipose tissue. Although present in other vertebrates, the gene encoding CHC22 is a pseudogene in mice. In this study, we demonstrate that CHC22 is associated with the glucose transporter GLUT4 and with components of its membrane trafficking pathway. In normal human skeletal muscle, CHC22 is localized to intracellular structures containing GLUT4 and in patients with type 2 diabetes, the co-localization between CHC22 and GLUT4 is even more obvious. Biochemical studies confirmed that CHC22 clathrin interacts with elements of the GLUT4 transport pathway. CHC22 down-regulation in cultured human myoblasts and adipocytes decreased GLUT4 levels and disrupted GLUT4 localization to its insulin-responsive storage compartment. CHC22 expression in the muscle of transgenic mice caused sequestration and aberrant localization of components of the GLUT4 transport pathway and decreased the amount of GLUT4 at the plasma membrane and T-tubules. In addition, these CHC22-transgenic mice, which also express CHC22 in adipose tissue, display features of type 2 diabetes including elevated fasting glucose levels and insulin resistance. These findings demonstrate a role for the clathrin heavy chain isoform CHC22 in formation and stabilization of the GLUT4 compartment in human insulin-responsive tissues. Furthermore, they highlight differences in glucose metabolism between humans and mice and have potential relevance to understanding mechanisms of human type 2 diabetes.

1472/B691

The Glucose Transporter Glut 4 Is Expressed in Retina.

R. Salceda[1], S. L. Baker-Reinoso[1], M. Peña- Rangel[2], J. R. Riesgo-Escovar[2]; [1]Neurociencias, Universidad Nacional Autonoma de Mexico, Mexico, D.F., Mexico, [2]Neurobiologia del Desarrollo y Fisiología, Universidad Nacional Autonoma de México, Juriquilla, Mexico

Glucose is the major fuel for energy metabolism in the retina. Glucose is transported into cells by a facilitative diffusion mechanism. Cloning and sequencing studies suggest the existence of several different glucose transport proteins for carrier mediated diffusion of glucose (Glut s) across cell membranes in various tissues. These transporters show different kinetic and regulatory properties. Insulin stimulates translocation of Glut 4 to the plasma membrane, increasing glucose accumulation. Retinopathy is one of the clinical manifestations of long standing diabetes mellitus. The regulatory mechanisms that maintain glucose homeostasis in retina may include hormonal action. Indeed, previous results suggest regulation of glucose uptake by insulin (Vilchis and Salceda, 1996) and insulin receptors have been reported in retina. Therefore, in this report we studied retinal expression of Glut 4 by immunohistochemical and in situ hybridization techniques. Immunoreactivity and in situ hybridization signals were observed in the ganglionar and inner nuclear layer as well as in the outer nuclear layer of the rat retina. The expression of Glut 4 transporter in retina can have significant relevance in retinal physiology and might have a role in diabetic retinopathy.

1473/B692

A Role for Calcium in the Insulin-Mediated Activation of Myosin IIA in 3T3-L1 Adipocytes.

Y. Patel, J. Brisson; Biology, University of North Carolina at Greensboro, Greenboro, NC

We have previously shown that insulin-responsive glucose transporter 4 (GLUT4)-mediated glucose uptake is a myosin II-dependent process in 3T3-L1 adipocytes. We also demonstrated that insulin stimulated phosphorylation of the myosin IIA isoform via myosin light chain kinase (MLCK) and that phosphorylation was required for myosin IIA to be recruited to the plasma membrane. The experiments described here reveal a role for calcium in activating myosin IIA during insulin-stimulated glucose uptake. We demonstrate that adipocytes treated with the calcium-specific chelator, 1,2-b is (o-a mino p henoxy) ethane-N,N,N',N'-tetra acetic acid, (BAPTA) impaired the insulin-induced phosphorylation of the regulatory light chain of myosin IIA. BAPTA also impaired the recruitment of myosin IIA to the plasma membrane as determined by confocal microscopy. In contrast, ethylene glycol tetraacetic acid, (EGTA) only slightly impaired myosin IIA recruitment to the plasma membrane after insulin stimulation. Furthermore, we show that thapsigargin, a Ca2+ATPase inhibitor specifically stimulated the phosphorylation of the RLC associated with the myosin IIA isoform and its subsequent recruitment to the plasma membrane, even in the absence of insulin stimulation. Collectively, our results are the first to demonstrate that calcium plays a critical role in activating myosin IIA during insulin-stimulated glucose uptake in 3T3-LI adipocytes, via regulating MLCK.

1474/B693

Hyperglycemia Enhances Transmission of Ca2+ Signals between Astrocytes.

M. M. Thi[1], M. Urban-Maldonado[1], D. C. Spray[1], S. O. Suadicani[2,1]; [1]Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, Bronx, NY, [2]Department of Urology, Albert Einstein College of Medicine, Bronx, NY

Diabetes increases the risk for stroke, and hyperglycemia significantly enhances the severity of ischemic injury. We have proposed that enhanced signaling between astrocytes and consequent astrocyte-neuron interaction may contribute to higher neuron loss in ischemic conditions. Astrocytes respond to various transmitters with increase in intracellular Ca^{2+}, release of "gliotransmitters" (i.e. ATP) and transmission of intercellular Ca^{2+} waves (ICWs). Two main pathways provide for this form of astrocytic Ca^{2+} signaling: an extracellular pathway mediated by released ATP and activation of purinergic receptors (P2Rs), and an intercellular gap junction-mediated pathway that allows direct cytosol-to-cytosol diffusion of generated second messengers. To investigate whether hyperglycemia enhances astrocytic ICW spread, primary mouse cortical astrocyte cultures were grown in control (CG: 1g/L or 5mmol/L) and in high glucose (HG: 4.5g/L or 25mmol/L) media and ICW spread was induced in Fura-2AM loaded cultures by focal mechanical stimulation of single cells and imaged by epifluorescence. Hyperglycemia significantly increased the amplitude (CG: 518±58nM, N=60 cells; HG: 809±79, N=73 cells, $P<0.01$), number of responding cells (CG: 27±2%; HG: 38±3%; N=5 fields, $P<0.05$) and radius of astrocytic ICW spread (CG: 120-160µm; HG: 180-200µm). This amplification of Ca^{2+} signaling between hyperglycemic astrocytes was likely associated with increased participation of $P2Y_1R$ and $P2Y_2R/P2Y_4R$ subtypes to ICW spread. Response of hyperglycemic astrocytes to bath application of UTP ($P2Y_2R/P2Y_4R$ agonist) was in average 2-fold higher than that of normoglycemic astrocytes, and the response to 2MeSADP ($P2Y_1R$ agonist) was 30% higher than normoglycemic controls. These findings indicate that astrocytic Ca^{2+} signaling is likely enhanced in diabetic brain and may contribute to higher sensitivity to ischemic injury in diabetes. Support: AHA 0735377N, NS041282, DK060037

1475/B694

Analysis of the ER Stress Response in an INS-1 Pancreatic Beta-Cell Clone Expressing an Inducible Folding Deficient Insulin 2 (C96Y) Gene.

T. S. Hartley[1,2], E. Lai[1,2], A. Volchuk[1,2,3]; [1]Cellular and Molecular Biology, Toronto General Research Institute, Toronto, ON, Canada, [2]Biochemistry, University of Toronto, Toronto, ON, Canada, [3]Physiology, University of Toronto, Toronto, ON, Canada

Cells respond to the accumulation of unfolded proteins during endoplasmic reticulum (ER) stress by activating the unfolded protein response (UPR), which increases chaperone capacity and enhances misfolded protein degradation. If ER stress is persistent then apoptosis is initiated, although how the cell decides when to commit to the apoptotic route is not known. ER stress can be induced by pharmacological agents that perturb ER homeostasis, but these typically cause intense stress conditions. To study the ER stress response in pancreatic beta-cells we developed a model system that allows for pathophysiological ER stress induction based on the 'Akita' mouse. This mouse strain expresses a mutant insulin 2 gene (C96Y), which prevents normal proinsulin folding, causing retention in the ER, ER stress, and eventual cell apoptosis. We developed a double-stable pancreatic beta-cell line (pTet-ON INS-1 Ins2(C96Y)-EGFP) with inducible Ins2(C96Y) expression. Electron microscopy revealed that the morphology of the ER of these cells becomes dilated by expressing the mutant insulin. Expression of the mutant insulin causes ER stress as measured by PERK and IRE1 pathway activation and expression of UPR target genes such as chaperones and

ERAD components. A time-dependent increase in apoptosis was observed up to 4 days of mutant insulin induction, although the levels of apoptotic cells were low. Blocking the proteasome during expression of the mutant insulin significantly increased the levels of apoptotic cells, indicating that the ERAD pathway is responsible for degrading the mutant insulin and enhancing cell survival. To identify gene expression changes following mutant insulin gene induction we performed microarray expression profiling experiments. In addition to the expected ER stress related genes, the gene with the highest level of induction was the Stromal Derived Factor 2-Like 1 (SDF2-L1). The function of this protein in the ER stress response is unknown and current work is attempting to define its role. This unique cell model of ER stress should allow for a detailed characterization of the UPR and potentially novel insights into the molecular mechanism of this process in pancreatic beta-cells.

1476/B695

Pdia Associates with Proinsulin in Pancreatic β Cells and Promotes Insulin Secretion.

M. Hiramoto, N. Maekawa, A. Nakanishi, T. Imai; Department of Aging Intervention, National Institute for Longevity Sciences, Obu, Japan

The point mutation (Ins2C96Y) found in the Akita diabetic mouse precludes formation of an essential disulfide bond between insulin chains and prevents proper folding and processing of proinsulin, and subsequent secretion of insulin. Therefore, the disulfide-bonds formation is very important process for proinsulin maturation. Objective: To elucidate the mechanism of the disulfide bonds formation of proinsulin2, we investigated the proteins that were associated with proinsulin2 in pancreatic β cells. Methods: We transfected pancreatic β cells with FLAG-tagged Ins2C96Y and prepared cell lysates 48 hours after transfection. We performed immunoprecipitation of the lysates using anti-FLAG antibody and analyzed the immunoprecipitates on SDS-polyacrylamide-gel electrophoresis and subsequent mass spectometry. Results: We identified mainly three proteins, heat shock 70kD protein 5 (Hspa5), protein disulfide isomerase associated (Pdia), and unnamaed protein product, which were specifically associated with FLAG-tagged Ins2C96Y. Next, we confirmed the association between proinsulin2 and Pdia using a specific antibody against insulin or Pdia. Finally, we showed that Pdia promoted the secretion of insulin2 when we co-transfected pancreatic β cells with insulin2 and Pdia. Conclusion: Pdia comprises thioredoxin-like catalytic domains and has been reported to have the isomerase activity *in vitro*. Taken together, these results suggest that Pdia is a key regulator for the disulfide-bonds formation of proinsulin2 in pancreatic β cells.

1477/B696

Angiotensin II Mediated Inhibition of Insulin Signaling Pathway Due to Angiotensin II Receptor-Insulin Receptor Interaction.

M. Ramdas[1], R. Kolhe[1], L. Pulakat[1], N. Gavini[1], A. Sved[2]; [1]Biological Sciences, Mississippi State University, Mississippi State, MS, [2]University of Pittsburgh, Pittsburgh, PA

Hypertension and insulin resistance are the leading factors for the pathophysiology of many other complications that encompass Metabolic Syndrome. Angiotensin II (Ang II), a multifunctional peptide responsible for regulation of blood pressure and fluid homeostasis is suggested to play a role in the pathogenesis of insulin resistance because inhibitors of Ang II biosynthesis or its receptor-AT1, improve insulin sensitivity. Previous studies have shown that downstream signaling molecules of the AT1 are involved in inhibiting Insulin Receptor (IR) signaling. To determine the role of receptor level interactions in this crosstalk, we tested for existence of direct interactions between the IR and Ang II receptors (AT1 and AT2). Previously we showed that Ang II-activation of the AT1 in human breast cancer cell line MCF-7 resulted in AT1-IRβ association and inhibition of insulin binding to IR. Here we show that Ang II-activation of the AT1 also causes differential phosphorylation of the IRβ in MCF-7. In the co-precipitation studies using lysates of heart tissues obtained from SD rats infused with Ang II and maintained on high salt diet, and spontaneously hypertensive rat (SHR), we show that both AT1 and AT2 interacts with the IRβ at a higher rate when compared to the lysates from tissues of normotensive rats. Thus, increased receptor level interaction between IRβ and Ang II-receptors exist in hypertensive heart. In addition, we observed that the rat AT1b and AT2 interact with the human IRβ subunit in yeast-two hybrid assay. The AT2 receptor spanning the amino acids 226-363 showed the strongest interaction. An AT2:AT1 chimeric receptor in which the third intracellular loop of AT2 was replaced with that of AT1 also interacted with the IRβ. However, a truncated AT2 receptor in which the cytoplasmic tail was deleted did not interact. Transient transfection of CHO cells with rat AT2 resulted in co-precipitation of the AT2 with IR-beta and inhibition of phosphorylation of IR-beta. Both the effects were reduced when CHO cells were transfected with AT2:AT1 chimeric receptor. Thus, the 3rd ICL and C-terminal region of the AT2 are involved in AT2-IRβ interaction and inhibition of IRβ phosphorylation.

1478/B697

Changes of Bone Morphogenetic Protein-7 (BMP-7) in the Recovery of Renal Function by Blood Sugar Control in Diabetic Rats.

C. Yeh, J. Cheng; Institute of Basic Medical Scineces, National Cheng-Kung Univesrity, Tainan, Taiwan

Background. Hyperglycemia increases the transforming growth factor (TGF-β) expression in kidney leading to diabetic nephropathy (DN). Exogenous bone morphogenetic protein-7 (BMP-7) may counteract TGF-β and protect renal damage. However, changes of renal BMP-7 by hyperglycemia relating to the progression of DN remain unclear. The present study investigated this point using streptozotocin-induced diabetic rats (STZ-DN) and cultured mesangial cells. Methods. After induction of type-1 like diabetes by STZ injection, rats develop DN after 9 weeks were termed as initial stage to compare with that after 13 weeks showed as established stage. We used insulin and phloridzin to correct hyperglycemia in these diabetic rats and measured their serum levels of glucose, blood urea nitrogen (BUN), creatinine, urine albumin excretion (UAE), and creatinine clearance (CrCl). Also, expressions of BMP-7, BMP-RII, TGF-β, and fibronectin were determined. The effect of glucose on BMP-7 and BMP-RII expressions were further characterized in cultured mesangial cells. Results. After insulin or phloridzin treatment, the levels of blood glucose and renal functions were markedly lowered in STZ-DN. Moreover, insulin or phloridzin increased BMP-7 and BMP-RII but decreased TGF-β and fibronectin expressions in kidney through correction of hyperglycemia in initial stage of STZ-DN. However, similar changes were not significant in established stage of STZ-DN. Expressions of BMP-7 and BMP-RII in mesangial cells were markedly reduced by high glucose. This decrease of BMP-7 in mesangial cells was reversed by blockade of TGF-β using antibodies. Also, BMP-7 expression in mesangial cells was inhibited by rhTGF-β and further reduced by high glucose after a longer of

incubation time. Conclusion. Control of hyperglycemia may improve renal functions and reverse renal BMP-7 and BMP-RII expressions at initial stage of DN but not at the established damage in type-1 like diabetic rats. Thus, earlier control of blood glucose will be helpful for the decrease of renal damage in diabetic disorders.

1479/B698

Effect of a High-fat Diet on Diabetic Mother Rats and Their Offsprings and Insulin Signaling.

R. Nasu[1], A. Nakamura[2], S. Kakarla[3], E. Blough[3], K. Kohama[2,3], T. Kohama[1]; [1]Departement of Health and Nutrition, Takasaki Univercity of Health and Welfare, Gunma, Japan, [2]Departement of Molecular and Cellular Phamacology, Gunma Univercity Graduate School of Medicine, Gunma, Japan, [3]Department of Biological Sciences, Marshall Univercity, Huntington, WV

Pregnant rats (first generation) were randomly divided into Diabetic (streptozotocin treated) and the control groups and fed either a control (7% fat in diet) or high-fat diet (21% fat in diet). Diabetic rats fed the high-fat diet were infertile, and first-generation and diabetic rats fed the control had a high stillbirth rate of 27.5±22.0% (mean±SE). In the first generation, the diabetic rats fed the control had a significantly lower body weight increase during the pregnancy than controls. The second-generation pup from diabetic mothers had a high blood glucose level at birth, and their triglyceride level was higher than that in the other two groups. The third-generation diabetic rats fed the control had a triglyceride level higher than that of control rats. Delivery was most difficult in diabetic rats fed the high-fat diet. Pups of diabetic rats fed the control diet exhibited growth retardation and increased blood glucose levels (240±13 mg/dl). We conclude that when the mother rat had diabetes, the next generation was also affected. We isolated the heart of diabetic and control rats and measured the expression and phosphorylation of Akt, one of the insulin signaling proteins. In the second-generation diabetic rats fed the control and high-fat diet, the phospholylation of Akt was decreased compared with that from control rats. The blood chemical findings of diabetic were showed the pups when the mother rats developed diabetes. In addition, the phosphorylation of Akt was decreased in the pups from the diabetic mother, and it was thought that the diabetic influenced the pups at cell level. We conclude that the feature of the diabetes was shown the next generation when the mother rats were diabetic.

1480/B699

Systemic Administration of MSC Prevents Renal Failure in Diabetic Mice.

F. Ezquer[1], M. Ezquer[1], V. Simon[1], D. Carpio[2], A. Yañez[2]; [1]Instituto de Ciencias Facultad de Medicina Clínica Alemana-Universidad del Desarrollo, Santiago, Chile, [2]Instituto de Bioquímica, Universidad Austral, Valdivia, Chile

Diabetes is one of the current main threats to public health. 25 to 40% of patients with diabetes develop diabetic nephropathy (DN). This syndrome comprises kidney failure and increased risk of cardiovascular disease. It represents the major single cause of chronic kidney disease and associated with premature morbi-mortality of diabetic patients. Up to date there is neither cure nor prevention of DN. It has been proved that mesenchymal stem cells (MSC) contribute to the regeneration of several tissues including acute injured kidney. Here we evaluated if MSC protect kidney function and structure when endovenously administered in a mice model of diabetes. For this, we induced diabetes in C57BL6 mice by a single injection of 200 mg streptozotocin (STZ) and animals presenting hyperglycemia, hypoinsulinemia, massive pancreatic islets destruction and low albuminuria, were separate in two groups. One group received two times 5x105 MSCGFP+ (day 30 and 51 post-STZ administration) and the other group received the vehicle. Whereas untreated diabetic mice gradually increased albuminuria, reaching values 15 times higher than normal animals, MSC-treated diabetic mice maintained low levels of albuminuria up to 4 months. When renal histology was studied, we observed that untreated diabetic mice had marked renal damage characterized by sclerosis and mesangial expansion at glomerular level and dilatation and proteins cylinders at tubular level. In contrast, in MSC-treated diabetic mice glomerular structures looked like normal and tubules showed only minor dilatation. The renoprotection observed after MSC administration was not associated to an improvement in pancreas function since during the whole follow up period both MSC-treated and untreated diabetic animals kept hyperglycemic, hypoinsulinemic and maintained few remnant beta-pancreatic islets. As we were able to detect GFP positive cells into the kidney of MSC-treated diabetic mice, we suggest that endovenously administered MSC home into diabetic kidneys, locally protect renal cells and therefore prevent renal failure in diabetic mice. Our results support MSC transplantation as a cell therapy strategy to prevent chronic renal diseases, in particular diabetic nephropathy.

1481/B700

Critical Roles of ATF3 on Streptozotocin-induced Diabetic Liver Injury in Mouse: ATF3 Mediates STAT1 Stabilization.

W. Kim, E. Song, J. Kim, S. Park; Center for Biomedical Sciences, Korea National Institute of Health, Seoul, South Korea

Background & Aims : It is well established that the administration of streptozotocin accelerates diabetic liver injury as well as type 1 diabetes, however the underlying mechanisms is not fully understood. Methods: The roles of ATF3 on STAT1 accumulation, which plays a critical role on diabetic liver injury induced by STZ administration, were investigated in STAT1-/- mice and ATF3 or STAT1-transfected cells. Results: STZ administration causes severe liver damage as evidenced by a significant elevation of serum ALT levels and apoptosis, correlated with the development of type I diabetes. STZ-induced apoptosis and liver injury were associated with the increase of STAT1, which was implicated in liver injury by virtue of its ability to promote hepatocyte apoptosis, but these events were strongly inhibited in genetic knock-out mice of STAT1 or IFN-γ. STAT1 overexpression was critically dependent upon ATF3, a stress-inducible gene, induced by STZ or IFN-γ because induced ATF3 did not changed in STAT1-/- mice and STAT1-siRNA or dominant negative STAT1-transfected cells, whereas induced STAT1 was significantly attenuated in cells containing inactive C-terminal-deleted ATF3(ATF3(ΔC)) or ATF3-siRNA. However, STAT1 activation was required for ATF3 translocation into the nucleus since ATF3 nuclear translocation did not observed in D/N STAT1- or STAT1-siRNA-transfected cells. Interestingly, STAT1 directly bound to the C-terminal domain of ATF3, which was confirmed by GST-pull down assay and co-IP assay. This direct interaction of ATF3 with STAT1 increased STAT1 stability by inhibiting its ubiquitination as well as proteasome activity. Conclusions: We showed that ATF3 functions as a potent regulator of STAT1 stability, which highly induced the liver injury via promoting apoptosis.

1482/B701

Analysis of the Activity of Some Naturally Occurring Protein Variants of the ATF6 Endoplasmic Reticulum Stress Sensor.

T. Teodoro[1,2], A. Volchuk[1,2]; [1]Toronto General Research Institute, Toronto, ON, Canada, [2]Department of Biochemistry, University of Toronto, Toronto, ON, Canada

Type 2 diabetes is a metabolic disorder characterized by insulin resistance and pancreatic beta-cell failure. Endoplasmic reticulum (ER) stress has been implicated as a potential molecular mechanism for the development of pancreatic beta-cell failure that occurs in type 2 diabetes. ATF6 is an ER-localized transmembrane protein that becomes activated by ER stress and translocates to the Golgi complex where it is proteolyzed, releasing a transcription factor. ATF6-p50 mediates gene expression changes associated with increasing ER chaperone capacity by inducing expression of GRP78 and other chaperones. Recently, single nucleotide polymorphisms (SNPs) in the ATF6 gene that result in amino acid substitutions have been associated with type 2 diabetes in some diabetic populations. Here, we tested the functional activity of some ATF6 variants (Pro-145-Ala) and (Ser-157-Pro) by expressing the active ATF6-p50 forms in HeLa cells. Transcriptional activity of the variant proteins was measured by a GRP78-promoter luciferase reporter assay, GRP78 mRNA induction and GRP78 protein expression. Results showed that there was no significant difference in transcriptional activity between the different variant proteins at positions 145 and 157. In addition, analyzing protein degradation rates of some of the protein variants by ^{35}S-methionine pulse-chase experiments showed no significant differences. Thus, it is seems unlikely that variations in these positions significantly affects ATF6 function, at least when overexpressed in Hela cells. While the Pro(145), Pro(157) variant has been associated with type 2 diabetes in the Pima Indian population (Thameem et al, 2006; Diabetes, 55:839), our results did not detect a significant difference in the activity of the various variants at these positions.

1483/B702

Nrf2 Activation Protects Cytokines- and Streptozotocin-induced B-Cell Damage by Suppressing the NF-Kb Pathway.

D. Ryu, K. Kwon, E. Kim, M. Song; College of Oriental Medicine, Wonkwang University, Iksan, Jeonbuk, South Korea

Sulforaphane (SFN) is an indirect antioxidant that protects animal tissues from chemical or biological insults by stimulating the expression of several families of Nrf2-regulated phase 2 enzymes. Treatment of RINm5F insulinoma cells with SFN dramatically decreased Keap1 protein level and increased PI3-K-dependent Nrf2 nuclear translocation. In addition, the phase 2 enzymes were also induced by SFN. Therefore, we evaluated whether the activation of Nrf2 by either SFN or ectopic overexpression of Nrf2 could inhibit cytokines-induced β-cell damage. Treatment of RIN cells with IL-1β and IFN-γ was found to induce β-cell damage through NF-κB-dependent signaling pathway. However, activation of Nrf2 by prior treatment with SFN prevented cytokines toxicity. In addition, the induction of Nrf2 overexpression by transfecting Nrf2 could also mimic the effects of SFN. The mechanism by which Nrf2 activation inhibited NF-κB-dependent cell death signals appeared to involve the reduction of oxidative stress, which was demonstrated by the inhibition of cytokines-induced H2O2 production. A protective effect of SFN was further demonstrated by assaying for normal insulin secreting responses to glucose in isolated rat pancreatic islets. In addition, pretreatment of mice with SFN blocked the development of type 1 diabetes. Key words: Nrf2, sulforaphane, NF-κB, β cells, cytokine, streptozotocin, phase 2 enzyme, ROS

1484/B703

Lectin-Based Enrichment of Variants of Dipeptidylpeptidase IV (DPPIV).

T. Geoui[1], A. Weiss[2], P. Porschewski[2]; [1]Global Product Management, Qiagen, Hilden, Germany, [2]R&D, Qiagen, Hilden, Germany

The aim of this study was to use a fast targeted biochemical approach involving lectin-affinity chromatography as a pre-analytical sample preparation step for the enrichment of N-glycosylated proteins and to screen for changes/differences (e.g., in the degree of sialylation of glycoproteins) that are physiologically and disease-relevant. For further characterization, glycosidase treatment, ELISA, and Western blot were used. Lectin-affinity chromatography allowed fast and simple enrichment and separation of glycoproteins of low abundance and depletion of non-glycosylated serum proteins (especially those leaking from tissue). Dipeptidylpeptidase IV (DPP IV) is a protein that has been associated with several diseases including type 2 diabetes and is known to cleave a wide range of physiological peptides. DPP IV was used as a model protein in this study to demonstrate the efficacy of the reported methodology for the enrichment of specific subsets of glycoproteins from healthy and diseased specimens on the basis of their glycan moieties and alterations in the chemical composition of their glycans. Serum levels of DPP IV from diabetes type 2 and non-diabetes specimens were assessed by immunoassay/ELISA and no differences were found. Differences between the groups were obtained by the measurement of the enzymatic activity of DPP IV in serum and the chromatographic distribution of DPP IV in the fractions obtained after lectin-affinity chromatography. We conclude that changes in the separation/fractionation efficacy of certain glycoproteins could contribute to the discovery of glycoproteins (e.g., sialylated glycoproteins) related to diseases.

1485/B704

Analysis of Hepatic PP1 Glycogen Targeting Subunits.

A. Danos, M. J. Brady; Medicine, University of Chicago, Chicago, IL

PTG and GL are hepatic protein phosphatase-1 (PP1) glycogen-targeting subunits, which target PP1 activity against glycogen synthase (GS) and phosphorylase (GP). To explore the specific roles of PTG and GL in glycogen metabolism, we focused on the high affinity GP binding site on the C-terminal end of GL, which is known to play a regulatory role. We generated a chimeric PTG-GL construct by cloning the 17 amino acid GP binding site from GL onto the 3' end of PTG. In addition, we removed the GP binding site from GL, generating GLtr. As expected, GP binding to GST-PTG-GL was increased 2-3 fold vs. GST-PTG, while GP binding to GST-GLtr was ablated. PP1 and GS binding were equivalent between GST-PTG and GST-PTG-GL. In vitro PTG-GL directed PP1 activity against GP was 55% of that directed by PTG. There was no difference between GL and GLtr directed PP1 activity against GP. In adenoviral overexpression studies of the 4 proteins in primary mouse hepatocytes, we observed that in cells overexpressing PTG, GS activity was elevated approximately 3 fold over untreated cells, but in PTG-GL cells, GS activity was 4 fold over untreated cells. In cells overexpressing GL, GS activity was approximately 4 fold higher in GL cells and 6 fold higher in GLtr cells than in untreated. GP activity assays were performed, and cells expressing PTG had

GP activity reduced to 50% of untreated, whereas PTG-GL cells had activity reduced to approximately 65% of untreated cells. GL expressing cells had GP activity reduced to 75%, and GLtr cells had 60% activity of untreated. Glycogen levels in PTG cells were at least 10-fold higher than untreated cells, whereas PTG-GL cells had glycogen levels approximately 60% of PTG cells. GL cells had glycogen levels at least 10-fold higher than untreated, whereas GLtr cells had 125% of GL treated glycogen. Under conditions promoting glycogenolysis, cells overexpressing GL and PTG-GL were able to mount a glycogenolytic response similar to that observed in wild type, but cells overexpressing PTG and GLtr did not effectively break down glycogen. These data suggest a role for GP in regulating GS activation, and also a novel role for the GP binding site in effective hepatic glycogenolytic response.

1486/B705

Mouse Models for Glycogen Storage Disease Type III and IV.

Y. Lee[1], C. Yang[1], J. Wu[1], Y. Chen[1,2]; [1]Institute of BioMedical Science, Taipei, Taiwan, [2]Pediatrics, Duke University Medical Center, Durham, NC

Glycogen storage disease type III (GSD-III) and IV (GSD-IV) are autosomal recessive diseases resulting from deficient glycogen debranching enzyme (AGL) and glycogen-branching enzyme (GBE) activity respectively. GSD-III is characterized by excessive accumulation of abnormal glycogen with short outer chains, in the liver and/or skeletal and cardiac muscles, with great variability in resultant organ dysfunction. GSD-IV is a clinically heterogeneous disorder characterized by the accumulation of an amylopectin-like polysaccharide. The typical presentation is liver disease of childhood, progressing to lethal cirrhosis. The neuromuscular form of GSD-IV varies in age of onset (perinatal, congenital, juvenile, or adult) and clinical severity. Using a gene-driven ENU-mutagenesis approach, the mice carrying the missense mutation V1387E in AGL gene or the stop codon mutation E609X in GBE1 gene have been generated. The mice carrying the homozygous V1387E mutation in AGL gene appeared normal at birth, however, growth retardation and hind limbs weakness soon developed at two weeks of age. The mice carrying the homozygous E609X stop codon mutation in the GBE1 gene showed perinatal death. The heartbeats of some homozygous mutation fetus stopped at around 10.5 days of gestation and histopathology revealed massive accumulation of glycogen in the myocardium of the common ventricular chamber, the main bronchus within the lung bud, and in the lumen of the stomach. These mice represent important animal models for the study of abnormal glycogen metabolism and its related toxicity and to investigate pathophysiology and treatment strategies for human GSD-III and GSD-IV.

Other Diseases II (1487-1499)

1487/B706

Effects of HIV Protease and Nucleoside Reverse Transcriptase Inhibitors on Macrophage Cholesterol Accumulation in Humans.

T. M. Guerin[1], R. N. Greenberg[2], J. White[1], H. O. Ballard[1], E. J. Smart[1]; [1]Pediatrics, Kentucky Pediatrics Research Institute, University of Kentucky, Lexington, KY, [2]Infectious Disease, University of Kentucky, Lexington, KY

The clinical data suggest that at least some HIV protease inhibitors may promote accelerated atherosclerosis. We have demonstrated that several HIV protease inhibitors, including ritonavir, amprenavir, and indinavir can induce atherosclerosis in a mouse model by up-regulating the scavenger receptor, CD36, in macrophages. Importantly we have also demonstrated that the nucleoside reverse transcriptase inhibitors didanosine and D4T prevent HIV protease inhibitor induced atherosclerosis by ubiquitination and degradation of protein kinase C. The purpose of the current study is to determine the effects of HIV protease inhibitors and nucleoside reverse transcriptase inhibitors on macrophages in healthy human volunteers. Non-HIV infected, healthy adult volunteers were given either three Kaletra capsules BID for 28 days or two Kaletra tablets BID for 28 days followed by another 28 days with Kaletra and Videx-EC 250 mg daily. Peripheral blood monocytes (PBMs) were isolated at two week intervals and analyzed for cholesteryl ester, CD36, and PPARγ content as well as PKC activity. Kaletra induced a 10-14 fold increase in cholesteryl ester content of PBMs that returned to normal two weeks post-Kaletra treatment. Importantly, macrophage free cholesterol levels did not change. In congruence, with the cholesteryl ester data, Kaletra induced an increase in CD36 protein levels (10-fold) and in PPARγ mRNA levels (18-fold) that returned to normal within two weeks after the removal of Kaletra. Videx co-treatment dramatically decreased Kaletra-induced increases in cholesteryl ester (2.3 fold), CD36 (1.8 fold), and PPARγ (2.1 fold). In addition, Videx induced an 87% reduction in the levels of PKC. These data demonstrate that the HIV protease inhibitor, Kaletra, can increase the expression of macrophage CD36 resulting in excessive accumulation of cholesteryl ester which could promote the formation of atherosclerotic lesions. In addition, co-treatment with Videx (didanosine) greatly limited the negative impact of Kaletra on macrophage cholesteryl ester accumulation. These data suggest a possible mechanism to explain the negative and variable impact of HAART (highly active anti-retroviral therapy) on cardiovascular health.

1488/B707

HNE-Induced 5-Lipoxygenase Activity Mediated MMP-9 Production through LTB4 and LTC4 Receptors in Murine Macrophages.

C. Kim, S. Lee, K. Seo, M. Yun, C. Kim; Pusan National University, Pusan, South Korea

4-Hydroxylnonenal (HNE), a factor proposed to be atherogenic, accumulates in atherosclerotic lesions, but its pathogenic role in the progression of atherosclerosis is not clear. Based on the importance of matrix metalloproteinases (MMP) in plaque destabilization and 5-lipoxygenase (5-LO) converts arachidonic acid to leukotrienes, which are potent proinflammatory lipid mediators. Thus, this study was designed to investigate the role of 5-LO in HNE-induced MMP-9 production in murine macrophages. HNE enhanced MMP-9 production and activity in a concentration- and time-dependent manners. Stimulation of macrophages with HNE led to an increased LTB4 production in association with an increased 5-LO expression. HNE-enhanced MMP-9 production and activity were significantly attenuated not only by MK886, a FLAP inhibitor but also by 5-LO siRNA. These results suggest that 5-LO plays a pivotal role on HNE-induced MMP-9 production. In addition, the leukotriene LTB4 receptor antagonist, U-75302 and the cysteinyl- LT receptor antagonist, REV-5901 inhibit HNE-induced MMP-9 production in a concentration-dependent manner, but not by the LTD4 receptor antagonists, MK-571. To assess whether autocrine release of LTB4 and LTC4 is involved in HNE-induced MMP-9 production, we determined the effects of LTB4 and LTC4

on HNE-induced MMP-9 production. These results indicate that HNE induces MMP-9 production through 5-LO-dependent autocrine secretion of LTB4 and LTC4. These results show that the interaction between LTB4 and LTC4 with its respective receptor is involved in MMP-9 production. Taken together, it is suggested that HNE-induced 5-LO activation and secretion of LTB4 and LTC4, which elicit production of MMP-9 through LTB4 and LTC4 receptors.

1489/B708

Sp1 Contributes HNE-Induced 5-Lipoxygenase Transcription Regulation Mediated MMP-9 Production in Murine Macrophages.

S. Lee, C. Kim, K. Seo, M. Yun, C. Kim; Pusan National University, Pusan, South Korea

4-hydroxynonenal (HNE) and 5-lipoxygenase (5-LO) are present in atherosclerotic lesions, HNE also induced MMP production and activity. Thus, the present study was designed to investigate the possible role of 5-LO expression in HNE-induced MMP-9 production. HNE enhanced MMP-9 activity in murine macrophages in association with an enhanced expression of MMP-9 mRNA and protein. Stimulation of macrophages with HNE also led to an increased 5-LO expression. HNE-enhanced MMP-9 production and activity were significantly attenuated not only by MK886, a FLAP inhibitor but also by 5-LO siRNA. These results suggest that 5-LO plays a pivotal role on HNE-induced MMP-9 production. To gain insights of transcriptional regulation of 5-LO gene, we examined the promoter activity of 5-LO. We have amplified a genomic sequence of promoter 2.0 kb upstream of the transcription initiation site of mouse genomic DNA by PCR. A series of sequentially deleted fragments were fused to pGL3 vector containing a luciferase reporter gene. Luciferase assay was performed by transfecting the constructs into a murine macrophages cell line, J774A.1. The assay resulted in the identification of promoter region 50-350 bp upstream of transcription start site, which is responsible for a remarkable enhancement of transcriptional activity in response to HNE. A site-directed mutagenesis of this region revealed that transcriptional factors were associated with up-regulation of 5-LO expression. Sp1 binding sites are critical for HNE-induced 5-LO transcription regulation. The role of Sp1 in 5-LO expression was further confirmed by knockdown experiments using siRNA of Sp1. These results suggest that Sp1-dependent HNE-induced 5-LO expression plays an important role in the MMP-9 production.

1490/B709

Cardiovascular Disease and Heat Shock Proteins (Hsps).

E. Dulin[1], M. Desco[2], M. Guisasola[2]; [1]Clinical Biochemistry Dep., Hospital General Universitario Gregorio Maranon, Madrid, Spain, [2]Experimental Medicine & Surgery Unit, Hospital General Universitario Gregorio Maranon, Madrid, Spain

Atherosclerosis is a chronic inflammatory disease, elevating intracellular levels of Hsp72 have anti-inflammatory properties, and antibodies (Abs) and Hsps have been related with the development and prognosis of atherogenesis. We studied the relationship between serum and neutrophil polymorphonuclear leukocytes (PMNs) [Hsp72], antiHsp72 and antiHsp60 antibody levels, with the risk of develop atherosclerosis. 100 women (age 49,21 +/-7,45) and 88 men (age 49,72 +/- 8,23) were studied randomly. Task Force Chart was applied for classification, and subjects divided into 3 groups: G0 (without vascular risk factors) [n=91 (63 women, 28 men), G1 with moderate vascular risk factors (VRF) (≥10%), without illness [n= 79 (32 women and 47 men)], and G2 with evident atherosclerotic (AT) illness [n=18 (5 women, 13 men)]. Male patients with VRF or AT, had the highest PMNs[Hsp72] and females with AT the lowest (p=0,068, Kruskas-Wallis test). As high PMNs[Hsp72] levels have anti-inflammatory properties, females with AT would have the worst prognosis when they develop the disease. Males trend to present an increase in circulating [Hsp72] in relation with females in all groups, especially in G1 (P=0,082); this constitute another worse prognostic factor in women with AT. Abs-Hsp72 levels trends to decrease in G1 and G2.All patients with AT present both reduced levels of serum [Hsp72] and AbsHsp72, what could be explained by the formation of immunocomplexes in the atheroma plaque, which would support the hypothesis of an autoimmune response to Hsps in the anti-inflammatory pathogenesis of AT. Males have the highest values in AbsHsp60, in all groups (p=0,009 G1; p=0,026 G2); their role in AT etiopathogenesis, might consider them biomarkers of development disease and predictor factors of independent VRF. The quantification of intracellular, serum Hsps and AbsHsps might be usefull to predict evolution to atherosclerosis. Grant from Fundación Mutua Madrileña.

1491/B710

Comparison of Effects with Respect to Diuretics on Intracellular Calcium Dynamics in Rat Testicular Arteriole Smooth Muscle Cells.

Y. Tamagawa[1,2], M. Matsuura[2], T. Saino[2], Y. Satoh[2]; [1]Pharmacy, Iwate prefectural Kuji hospital, Kuji, Japan, [2]Anatomy (Cell Biology), Iwate Medical University, Morioka, Japan

[OBJECTIVE] A diuretic is used for antihypertension to reduce circulatory blood volume, in many cases. Somehow we imaged that in the cases blood vessels relaxed, but exactly, it is uncertain that the reduction in blood pressure is due to decreased blood volume resulting from increased urine production. Arterioles play pivotal roles in controlling blood pressure and regional circulation in any organs. Here, we have investigated effect of various diuretics on intracellular calcium ion concentration ($[Ca^{2+}]i$) dynamics which is an essential factor of smooth muscle contraction. **[METHODS]** We isolated arteriole specimens keeping their fundamental histological structures from testis of rats. They were soaked in Hepes-buffered Ringer's Solution (HR) (pH7.4). Connective tissues around arterioles were digested by purified collagenase (100 units/ml), then the specimens were loaded with Indo-1/AM (5 µM, 30 min, 37°C; a Ca^{2+} indicator) . They were placed on cover slides in chambers which were coated with Cell-Tak and continuously perfused with HR containing diutetics; hydrochlorothiazide (HYD), furosemide (FUR) and spironolactone(SP). We used a real-time confocal microscope (Nikon RCM/Ab) to observe $[Ca^{2+}]i$ dynamics. **[RESULTS]** HYD and FUR had little effects on [Ca2+]i. When SP (500 µM) was used, an initial increase and following oscillatory fluctuations of [Ca2+]i in the smooth muscle cells were observed. Apparent contraction of arterioles was elicited. The response was partially inhibited when extracellular Ca^{2+} was removed from perfused HR. Thapsigargin, an inhibitor of the ER Ca2+ pump, has few effects on SP-induced $[Ca^{2+}]i$ dynamics. Neuro-toxic tetrodotoxin did not affect on SP-induced $[Ca^{2+}]i$ dynamics, indicating that the SP effect was not caused by nerve fibers around the vessels. **[CONCLUSIONS]** The SP can increase of $[Ca^{2+}]i$ in rat arteriole smooth muscles mainly via Ca^{2+} influx from extracellular space. Although SP has been commonly used for hypertension and/or edema, we have to pay attention to possibility of temporal increase of blood pressure.

1492/B711

ATP-Induced Intracellular Calcium Dynamics in Prostatic Smooth Muscles of Golden Hamsters.

M. Matsuura[1,2], T. Kuroda[2], T. Saino[2], Y. Satoh[2], Y. Sadzuka[1]; [1]Advanced Pharmaceutics,School of Pharmacy, Iwate Medical University, Morioka, Japan, [2]Anatomy (Cell Biology), Iwate Medical University, Morioka, Japan

[OBJECTIVE]The prostate is a compound tubuloalveolar exocrine gland of the male mammalian reproductive system. The prostate surrounds the urethra just below the urinary bladder and contains contractile stroma cells (so called, smooth muscles, although the structure and function can differ from vascular or intestinal smooth muscles) that help expel prostatic contens in semen. Benign prostatic hyperplasia (BPH) occurs in older men; the prostate often enlarges to the point where urination becomes difficult. It is well known that prostatic smooth muscles have adrenergic receptors. Until now, some blockers of these receptors are widely used for BPH. However, under the present circumstances these drugs don't have enough effects. It is well known that ATP is released from adrenergic nerve terminals. Here, we study the effect of ATP in prostate smooth muscle cells of golden hamsters using an imaging technique. [Method] We used male Golden hamsters (120-140 g). Prostatic gland endpieces were isolated and soaked in Hepes-buffered Ringer's Solution (HR) (pH7.4). Connective tissues were digested by purified collagenase (100 units/ml) for 1 hours at room temperature. Then the specimens were loaded by Indo-1/AM (5 μM) for half hours at room temperature. They were placed on cover slides in chambers and continuously perfused with HR containing ATP and/or some specific analogs. We used a real-time confocal microscope (Nikon RCM/Ab). [Results] When ATP (100 μM) was used as a stimulus, an increase of [Ca2+]i in the smooth muscle cells was observed. This response was abolished when extracellular Ca2+ was removed from perfused HR. However in the same condition, noradrenaline (NorAdr)- induced Ca2+ increase is partially inhibited. The pattern of [Ca2+]i increase is different between ATP and NorAdr; such as a spike-shaped change, a rhythmic change and persistent elevation. [Conclusion] In present study, we propose that the major pathway of ATP-induced [Ca2+]i increase in prostatic smooth muscle cells is a Ca2+ influx via P2X receptors. And we suggest that these cells have different reaction mechanisms between ATP and NorAdr. There is some possibility that antagonists of P2X receptors are effective for BPH.

1493/B712

Galectin-1 Is Highly Expressed in Dystrophic and Injured Skeletal Muscles.

M. R. Costa[1,5], D. G. Cerri[1], L. C. Rodrigues[1], S. R. Stowell[3,4], D. Araujo[1,5], M. C. Coelho[1], S. R. Oliveira[1], J. S. Bizario[1], R. D. Cummings[3,4], M. Dias-Baruffi[2]; [1]Muscular Dystrophy Research Center AADM/UNAERP, University of Ribeirao Preto, Ribeirao Preto, Brazil, [2]Analises Clinicas, Toxicologicas e Bromatologicas - FCFRP, University of Sao Paulo, Ribeirao Preto, Brazil, [3]Biochemistry and Molecular Biology - Oklahoma Center for Medical Glycobiology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, [4]Biochemistry, Emory University School of Medicine, Atlanta, GA, [5]Cellular and Molecular Biology - FMRP, University of Sao Paulo, Ribeirao Preto, Brazil

Galectin-1 (Gal-1), a 14 kDa beta-galactoside binding lectin, plays crucial roles in processes such as cell adhesion, apoptosis, phagocytosis, inflammatory events, immune reactions, cell proliferation/differentiation. Recent studies showed that Gal-1 increases skeletal muscle differentiation and regeneration, suggesting its potential involvement in neuromuscular diseases like Duchenne Muscular Dystrophy (DMD), a fatal and still incurable disease. Understanding the mechanisms and factors responsible for muscle degeneration and regeneration will facilitate the development of novel therapeutics. However the expression and localization of Gal-1 in vivo during muscle injury and repair is unclear. We report the expression and localization of Gal-1 during degenerative-regenerative processes in vivo using two models of muscular dystrophy and muscle injury. Gal-1 expression increased significantly during muscle degeneration in the murine mdx and in the canine Golden Retriever Muscular Dystrophy animal models. Compulsory exercise of mdx mice, which intensifies degeneration, also resulted in sustained Gal-1 levels. Furthermore, muscle injury of wild type C57BL/6 mice, induced by BaCl2 treatment, also resulted in a marked increase in Gal-1 levels. Increased Gal-1 levels appeared to localize both inside and outside the muscle fibers with significant extracellular Gal-1 co-localized with infiltrating CD45+ leukocytes. By contrast, regenerating muscle tissue showed a marked decrease in Gal-1 to baseline levels. These results demonstrate significant regulation of Gal-1 expression in vivo, and suggest a potential role for Gal-1 in muscle homeostasis and repair.

1494/B713

Mechanical Stress Induced Myofibrillar Disruption Causes Cardiomyopathy in Bag3 Deficient Mice.

A. Hishiya, S. Takayama; Boston Biomedical Research Institute, Watertown, MA

<Objective> Bcl-2 associated athano gene 3 (BAG) is a member of the co-chaperone BAG family proteins that binds to and regulates Hsp70 molecular chaperones. The bag3 deficient mice develop muscle degeneration usage dependently. The underlying mechanisms, however, have remained elusive. We established in vitro system using rat cardiomyocytes to address the effect of mechanical stress. <Methods> Equibiaxial stretch was applied to cardiomyocytes, which were infected with adenovirus carrying siRNA of bag3. <Results> Interestingly, in bag3 knockdown cardiomyocytes, mechanical stretch rapidly disrupted both F-actin and Z-disc structures. In bag3 deficient heart, enlargement of ventricle was observed with cytoskeletal disorganization. <Conclusions> The evidence indicates that BAG3 has important roles on the maintenance of myofibrillar structure under the mechanical stress and the failure leads to cardiomyopathy.

1495/B714

Quercetin Inhibits Ischemia-induced Cardiomyocyte Apoptotic Death through Inhibition of p38 Mitogen-activated Protein Kinase Pathway.

D. Lee[1,3], S. Park[1], M. Kim[1,3], S. Lee[1,2], E. Baik[1,2], C. Moon[1,2], Y. Jung[1,3]; [1]Department of Physiology, Ajou University, Suwon, South Korea, [2]Brain Korea 21 for Medical Science, Ajou University, Suwon, South Korea, [3]Brain Korea 21 for Molecular Science and Technology, Ajou University, Suwon, South Korea

Epidemiological studies suggest that the consumption of quercetin diets decreases the risk of cardiovascular diseases. However, the molecular mechanisms of quercetin on ischemic heart disease are still unclear. In the present study, we investigated the effect of quercetin on ischemic injury in heart-derived H9c2 cells in vitro, and further investigated the underlying mechanism. To evaluate the effect of quercetin

on ischemia-induced cell death, we measured LDH release and TUNEL-positivity in H9c2 cells. To investigate the mechanism of the cardioprotection by quercetin, we measured p38 MAPK phosphorylation (P-p38 MAPK) by western blotting in H9c2 cells. Quercetin produced a preventive effect against ischemia-induced apoptotic death in H9c2 cells. The quercetin significantly inhibited the p38 MAPK phosphorylation during hypoxia in H9c2 cells. In conclusion, the results from this study suggest that quercetin exerts a significant cardioprotective effect against ischemic injury, at least in part, through the inhibition of p38 MAPK phosphorylation. This study was supported by "Specific Joint Agricultural Research-promoting Projects (20080401-033-302-001-02-00)", RDA, Republic of Korea.

1496/B715

The Role of GATA-6 in the Pathogenesis of Pulmonary Arterial Hypertension.

A. Ghatnekar, M. Trojanowska; Medical University of SC, Charleston, SC

Background/Objective: Pulmonary arterial hypertension (PAH) is an increase in blood pressure in the pulmonary artery or lung vasculature. The key features of this disease are vasoconstriction, followed by intimal proliferation and fibrosis, in-situ thrombosis, and plexogenic changes. Several pathways are known to be perturbed in endothelial cells during PAH: TGF-β/BMP, nitric oxide (NO), and endothelin. Interestingly, a recent study revealed that GATA6, a zinc finger transcription factor, is downregulated in lung tissue of a rat PAH model. The objective of this study is to determine whether GATA6 is involved in the regulation of gene programs altered in PAH. Methods: We first performed a microarray analysis of human endothelial cells (ECs) with AdsiRNA-mediated GATA6 knockdown, and subsequently validated putative target genes by qRT-PCR. We also investigated the potential role of GATA6 as a transcriptional repressor of Tenascin-C, an extracellular matrix protein that plays a pathological role in PAH, by using CAT reporter constructs and ChIP analysis. Lastly, we performed immunohistochemical (IHC) analysis of lung biopsies from Scleroderma patients with PAH to determine if Gata6 is differentially expressed in the pulmonary vasculature. Results: Among the genes altered in endothelial cells after suppression of GATA6 were components of the bone morphogenetic protein (BMP) pathway, BMPR2, BMP4 and BAMBI, as well as eNOS, MMP1, and MMP10. Both knockdown and forced expression of GATA6 in ECs confirmed that GATA6 might be a negative regulator of MMP-1 and MMP10, and a positive regulator of eNOS and BAMBI gene expression. We have also shown that overexpression of GATA-6 in fibroblasts inhibits both basal and cytokine-induced TNC protein and mRNA levels. A GATA6 response element was mapped in the proximal TN-C promoter region. Lastly, IHC analysis of lung biopsies from Scleroderma patients with PAH, revealed that GATA6 is dramatically reduced overall in vascular smooth muscle cells and endothelial cells, as well as endothelial cells in intimal proliferation. Conclusion: GATA6 may regulate genes responsible for vascular remodeling and therefore, loss of its expression may contribute to the development of PAH.

1497/B716

Novel Molecular Signatures for Human Pulmonary Arterial Hypertension.

V. Karamanian[1,2], M. Harhay[1,4], K. Ihida-Stansbury[1,2], M. Albuquerque[5,6], H. Palevsky[4], M. C. Tamby[1,2], A. Martinez Suiffet[7], D. Taichman[1,4], P. L. Jones[1,2,3]; [1]Penn-CMREF center for pulmonary arterial hypertension research, Univerisity of Pennsylvania, Philadelphia, PA, [2]Institute for Medicine and Engineering, Univerisity of Pennsylvania, Philadelphia, PA, [3]Pathology and Laboratory Medicine, Univerisity of Pennsylvania, Philadelphia, PA, [4]Medicine, Univerisity of Pennsylvania, Philadelphia, PA, [5]Pediatrics, Univerisity of Pennsylvania, Philadelphia, PA, [6]Children's Hospital of Philadelphia, Philadelphia, PA, [7]TodoSoft Uruguay, Montevideo, Uruguay

Many studies have attempted to understand the clinical and pathological conditions of pulmonary arterial hypertension (PAH); yet, it remains an incurable disease, in need of less invasive diagnostic tools and a better patient-specific therapy. To this end, we analyzed the plasma protein profile of 33 healthy volunteers and 113 PAH patients, 58 idiopathic (IPAH), 55 non-idiopathic (NIPAH), using multi-analyte profiling (MAP) of a commercial antigen array. Analysis of 100 ul of patient plasma identified 38 antigens whose levels were significantly ($p<0.05$) increased (n=33) or decreased (n=5) in PAH patients when compared to controls. These novel disease-associated antigens include: onco-fetal proteins, growth factors, pro-angiogenic molecules, stem cell factors, immune-inflammatory mediators, and components of the extracellular matrix. Importantly, 22 antigens were significantly increased or decreased between IPAH and NIPAH patients, potentially allowing for diagnosis of different forms of the disease. Significant differences were identified in the erythropoietin (EPO) hormone concentration of PAH patients, a known pro-angiogenic, anti-apoptotic molecule, when compared to controls (85 fold increase in IPAH, 147 fold increase in NIPAH vs control). Thus, future experiments will test the role of EPO, known pro-angiogenic molecule, as well as PAH patient-derived EPO in endothelial cell angiogenesis assays. In this study, novel circulating antigens from a large scale sample of PAH patients have been identified and quantified by MAP analysis, distinguishing not only PAH patients from control, but also IPAH patients from those with NIPAH. These novel results and technique provide new insights into the pathophysiology and diagnosis of PAH, in addition to identifying new therapeutic targets.

1498/B717

Contact of Peripheral Blood Mononuclear Cells from Psoriatic Patients with Collagen Generates a Pro-Inflammatory Cytokine Profile.

J. Sánchez-López[1,2], M. Baeza-Ramírez[2], F. Jurado-Santacruz[3], C. López-Macías[1], A. Isibasi[1], L. ARRIAGA-PIZANO[1]; [1]Unidad de Investigación Médica en Inmunoquímica, IMSS, Mexico, Mexico, [2]Laboratorio de Biomembranas, Escuela Nacional de Ciencias Biológicas IPN, Mexico City, Mexico, [3]Centro Dermatológico Ladislao de la Pascua, Mexico City, Mexico

Objective: To determine if the contact of peripheral blood mononuclear cells (PBMCs) from psoriatic patients with collagen type I or type IV increases the pro-inflammatory cytokine response, relative to non-psoriatic individuals. Material and Methods: PBMCs were isolated from peripheral blood from psoriatic patients (n=12) or healthy volunteers (n=10), and were incubated with or without collagen type I (Col I) or collagen type IV (Col IV) for 12h in a 24 well cell culture cluster. Cultures were stimulated or not with lipopolysacharide (LPS; 1μg/ml) for 4h. Supernatant was collected for IL-1β and IL-10 quantification using a cytokine bead array human inflammation kit. Results: Under control conditions (no LPS), PBMCs from patients with psoriasis produced more IL-1β than cells from their healthy counterparts (29.47±27.5 ng/mL vs. 15.87+20.14 ng/mL, respecitvely, p=0.0435). The higher secretion of IL-1β from PBMCs from psoriatic patients was of greater magnitude after LPS stimulation (48.13+46.52 ng/mL vs. 17.07+22.31 ng/mL, respectively; p=0.0133). Similarly, IL-1β production was

greater from cells from psoriatic patients than from cells from healthy individuals after stimulation with Col IV (90.07+59.47 ng/mL vs. 35.73+29.01 ng/mL, respectively; p=0.0435), after Col I stimulation (41.22+52.03 ng/mL vs. 13.12+12.17 ng/mL, respectively; p=0.0279), and after Col I-LPS stimulation (50.86+40.61 ng/mL vs. 14.16+9.911 ng/mL, respectively; p=0.0057). In contrast, PBMCs from psoriatic patiens released less IL-10 than cells from healthy individuals after stimulation with Col IV-LPS (1.293+1.834 ng/mL vs. 2.481+ 2.562 ng/mL, respectively; p=0.0394). Conclusions: These results suggest that contact with collagen during PBMCs infiltration to the skin could exacerbate the pro-inflammatory local response in psoriatic patients.

1499/B718

Role of Protein Kinase C in the Regulation of Plasminogen Activator Inhibitor-1 Gene Expression in Osteoarthritic Chondrocytes by Fluid Flow.

C. Yeh[1], L. Chen[2], C. Chen[2]; [1]Orthopaedic Department, Chiayi Veterans Hospital, Chiayi City, Taiwan, [2]Department of Biochemical Science and Technology, National Chiayi University, Chiayi City, Taiwan

Osteoarthritis (OA) is characterized by a progressive erosion of cartilage, which is mediated by the degradation of the extracellular matrix components. The plasminogen activator system is thought to play an important role in the regulation of the OA pathophysiology process. In addition, mechanical environment plays an important role in maintaining cartilage integrity. In this study, we investigated the effects of fluid shear stress in regulating the gene expressions of plasminogen activator inhibitor-1 (PAI-1) in human OA chondrocytes, and the roles of protein kinase C (PKC) in this regulation. The chondrocytes were subjected to fluid shear stress (2 ~ 20 dyn/cm2) or kept under static condition for various time periods (30 min ~ 4 h). 5 and 10 dyn/cm2 shear stress specifically caused significant up-regulations of PAI-1 gene expressions over the 4 h of study, and a transient activation of PKC was starting at 2 min and declining to basal level in 1 h. The shear stress-induced PAI-1 was blocked by pan-PKC inhibitor (bisindolylmaleimide I) and conventional PKC inhibitor (GO 6976). Analysis of the cis-elements in the PAI-1 promoter suggests the involvement of transcription factor Sp-1 in the gene expression regulation. The conventional PKC inhibitor and PKCα siRNA inhibited SP-1-DNA binding activity, as well as PAI-1 gene expression. These results suggest that the shear stress-induction of PAI-1 gene was mediated through the PKCα/Sp-1/PAI-1 pathway.

Symposium V: Nuclear Organization and Disease (1500-1502)

1500

RNP Biogenesis and Global Splicing Regulation.

G. Dreyfuss; Howard Hughes Medical Institute, University of Pennsylvania School of Medicine, Philadelphia, PA

Cells contain several different classes of RNAs (snRNAs, mRNAs, etc.) and an enormous assortment of RNA-binding proteins. RNAs exist in cells as RNA-protein complexes (RNPs) and each class (snRNPs, mRNPs, etc.) is associated with a specific subset of RNA-binding proteins. Although RNPs were believed to form by self-assembly, we found that the biogenesis of snRNPs, the major components of the pre-mRNA splicing machinery (the spliceosome), and likely other RNPs in eukaryotic cells, is carried out by the survival of motor neurons protein (SMN) complex. The SMN complex binds to the snRNA-binding proteins (the Sm snRNP proteins) as well as to snRNAs, which it recognizes by specific sequence features (the snRNP code) that distinguish them from other classes of RNAs. The purpose of the SMN complex is to confer stringent specificity to otherwise potentially illicit RNA-protein interactions. Although SMN is ubiquitously expressed and is required for viability of all cells, genetic lesions that result in functional SMN protein deficiency cause spinal muscular atrophy (SMA), a common motor neuron degenerative disease and a leading hereditary cause of infant mortality. SMA severity corresponds directly to SMN deficiency, which causes a commensurate decrease in the capacity for snRNP assembly. We recently found that SMN deficiency has drastic and cell type-specific effects on the repertoire of snRNAs and mRNAs. It alters the stoichiometry of snRNAs and causes widespread pre-mRNA splicing abnormalities in numerous transcripts of diverse genes in SMN-deficient mouse tissues. These surprising findings reveal a key role for the SMN complex in RNA metabolism and in splicing regulation, and indicate that SMA is a general splicing disease that is not restricted to motor neurons. We have utilized high throughput screening to discover compounds that modulate the activity, localization and amount of the SMN complex in cells, as research tools and potential therapeutics for SMA.

1501

Nuclear Lamins Control Adult Stem Cell Proliferation and Differentiation.

R. Foisner, N. Naetar, I. Gotic; Max. F. Perutz Laboratories, Medical University of Vienna, Vienna, Austria

Lamins are nuclear intermediate filament proteins in multicellular eukaryotes that form a scaffolding network at the nuclear envelope. While B-type lamins are ubiquitously expressed, A-type lamins are only expressed at later stages of development. Intriguingly, mutations in the LMNA gene, encoding A-type lamins, have been linked to human diseases, whose pathologies range from striated muscle defects to premature ageing syndromes. The molecular pathomechanisms, how mutations in lamins affect their functions and how these can give rise to the diverse pathologies are poorly understood. One disease hypothesis proposes that mutations impair lamin structure and stability leading to nuclear fragility, while another hypothesis postulates lamin-linked defects in chromatin organization and gene expression control in diseased cells. We and others recently discovered a novel function of lamins in cell cycle control of adult stem cells in regenerating tissues, which may be highly relevant for the disease phenotypes. Unlike the well-studied major lamin structures at the nuclear envelope, lamin A/C complexes involved in stem cell control are located in the nuclear interior. We found that a specific lamin A-binding protein, termed lamina-associated polypeptide 2 alpha (LAP2α), is required for the localization of a subfraction of lamin A/C in the nuclear interior. The intranuclear LAP2α-lamin A/C complex regulates retinoblastoma protein (Rb), a major cell cycle regulator protein involved in controlling proliferation and differentiation of adult stem cells. Knockdown of LAP2α in mice impaired the Rb pathway in postnatal regenerative tissues and caused accumulation of proliferating early progenitor cells in skin and intestine and of immature erythroblasts in the hematopoietic system due to an inefficient cell cycle arrest. Based on these findings, we propose that disease-causing mutations in lamins may disturb the balance between proliferation and differentiation of early progenitor cells, and impair tissue homeostasis in patients. This study was supported by the Austrian Science Research Fund and the EURO-Laminopathies research project of the European Commission (Contract LSHM-CT-2005-018690).

1502

How Shelterin Protects Chromosome Ends.

T. deLange; Laboratory of Cell Biology and Genetics, The Rockefeller University, New York, NY

Mammalian chromosome ends are protected by a protein complex, called shelterin, that binds specifically to telomeric TTAGGG repeats sequence. These repeats are only found at intact chromosome ends where they are maintained by telomerase. Shelterin contains three DNA binding proteins (TRF1, TRF2, and POT1) that confer exquisite specificity for TTAGGG repeats, hence ensuring shelterin's association with natural chromosome ends but not other DNA ends. We have used conditional deletion of shelterin components from mouse embryo fibroblasts to study the role of this complex in the protection of chromosome ends. Shelterin deficient telomeres activate DNA damage signaling pathway, leading to cell cycle arrest, and undergo processing by the two main DNA repair pathways, homology directed repair and non-homologous end-joining. The phenotypes associated with deletion of the individual shelterin components reveal how shelterin functions and provide insight into the mechanisms of DNA repair and DNA damage signaling.

Education Initiative Forum II: Implications for Hiring Cell Biology Faculty with Education Specialties: A Cross-Disciplinary Research Study of Science Faculty with Education Specialties (SFES) in the California State University System (1503)

1503

Implications for Hiring Cell Biology Faculty with Education Specialties: a Cross-Disciplinary Research Study of Science Faculty with Education Specialties (SFES) in the California State University System.

K. D. Tanner[1], S. D. Bush[2], N. J. Pelaez[3], J. A. Rudd[4], M. T. Stevens[5], K. S. Williams[6]; [1]Biology, San Francisco State University, San

Francisco, CA, [2]Chemistry and Biochemistry, California Polytechnic State University, San Luis Obispo, San Luis Obispo, CA, [3]Biological Sciences, Purdue University, Purdue, IN, [4]Chemistry and Biochemistry, California State University, Los Angeles, Los Angeles, CA, [5]Biological Sciences, California State University, Stanislaus, Turlock, CA, [6]Biology, San Diego State University, San Diego, CA

Efforts to improve K-16+ science education in the U.S. have included the hiring of Science Faculty with Education Specialties (SFES) - scientists who take on specialized roles in science education within their departments, including biology departments (Bush, 2006). Though SFES occupy a potentially pivotal role in reform efforts, there has been little systematic investigation of these positions. We present the first cross-disciplinary research study of SFES in the U.S., conducted in the nation's largest university system, the California State University (CSU). Of 156 CSU faculty identified as likely SFES, 103 individuals participated in a 111-question online survey (66% response rate). Of those respondents who both were tenure-track science faculty and submitted complete surveys, 59 self-identified as SFES, representing biology (34%), chemistry (24%), geoscience (14%), and physics (25%). They were 46% female, 81% white, across all faculty ranks, and trained extensively as researchers in basic science. Two SFES subpopulations were identified, those specifically Hired (H-SFES; n=31, 53%) as SFES and those who Transitioned (T-SFES; n=28, 47%) from their initial faculty roles into science education. Interestingly, more T-SFES (75%; $\chi 2$ =4.4, df=1, p=0.036) reported pursuing basic science research funding compared to H-SFES (48%), whereas more H-SFES (68%; $\chi 2$ =2.7, df=1, p=0.098) applied for science education research funding compared to T-SFES (46%). Both groups applied at equal rates (68%) for funding to support K-12 teacher development. Both SFES populations reported more formal training in basic science than in science education, more time spent on service responsibilities than non-SFES science faculty, and less institutional support for their scholarly activities. Unfortunately, almost 40% of SFES were seriously considering leaving their position, with H-SFES citing that science education efforts were not valued or understood and T-SFES citing being overworked or burned out. These research findings can inform biology departments considering SFES faculty hires and also indicate a need for national leadership to articulate expectations and establish training pathways for future SFES.

Symposium VI: Cytoskeletal Dynamics (1504 – 1506)

1504

The Role of the Cytoskeleton in Cell Mechanics and Plasticity.

M. Plodinec[1], R. Zanetti-Dallenbach[2], S. Eppenberger[3], C. Schoenenberger[1], U. Aebi[1]; [1]M.E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Basel, Switzerland, [2]Women's Hospital, University of Basel, Basel, Switzerland, [3]Stiftung Tumorbank, Basel, Switzerland

Cell plasticity plays a major role in many physiological and pathological processes, including embryonic development, tissue damage and repair, as well as tumor progression. By sensing and integrating signals from the environment, the actin and intermediate filament (IF) cytoskeleton are both primary determinants/regulators of cell and tissue plasticity. In order to understand how these two dynamic filament systems contribute to mechanical responses of cells and tissues in physio-pathological situations, we record stiffness maps of living cells and tissues by identation-type atomic force microscopy (IT-AFM). Because altered cell stiffness is a characteristic of cancer and many other diseases, its correlation with structural and functional changes of the actin and IF cytoskeleton might open new vistas for therapeutic intervention. The effect of disease causing mutations in actin and IF proteins on cell stiffness were examined in different cell lines grown in 2D and 3D culture systems: fibroblasts expressing a mutant actin gene which has been shown to render cells tumorigenic; SW-13 carcinoma cell subclones that lack or contain endogenous vimentin; and cells expressing mutant desmins shown to be associated with myopathies. The resulting stiffness maps were correlated to histological, morphological and biochemical parameters. IT-AFM revealed marked differences in the nanomechanical properties of cells with altered cytoskeletons, provided cells were grown as 3D spheroids. This emphasizes the fact that the tissue architecture is a primary determinant of the mechanical response. Data on the mechanical properties of cells from cancer patients have recently been obtained from 2D cell culture systems. With the evidence increasing that the nanomechanical properties are a signature of cancer cells and thus may serve as a hallmark to identify the early onset of tumor progression, the characterization of the elastic properties of 3D cancer tissues gains significance. Data from a pilot study, where the nanomechanical properties of paired, malignant and adjacent non-malignant tissue biopsies from breast cancer patients were mapped by IT-AFM, will be discussed.

1505

Regulation of Microtubule Dynamics and Organization during Spindle Assembly.

C. Walczak; Department of Biochemistry and Molecular Biology, Indiana University, Bloomington, IN

Regulated dynamics of microtubules are critical for the organization of the cytoskeleton and for the assembly of the mitotic spindle, which is utilzed to segregate chromosomes to the two daughter cells. Proper spindle dynamics require the action of the microtubule depolymerizing kinesins, including MCAK, whose activities are regulated both spatially and temporally within the spindle. Inhibition or knockdown of MCAK disrupts spindle organization by dramatically altering the distribution of microtubules between the astral array and the spindle. Once a spindle is assembled, dynamic microtubules are critical to maintain spindle organization and to couple the spindle to the dynamic movements of chromosomes during congression and segregation. It is widely accepted that congression requires chromosomes to be attached to the K-fiber, which is a bundle of microtubules that attaches to the kinetochore in an end-on manner. We found that chromosomes in human cells can efficiently congress to the metaphase plate in the absence of K-fibers. We disrupted K-fibers through knockdown of the microtubule attachment protein hNuf2 and then identified a condition that restored chromosome alignment. Simultaneous knockdown of hNuf2 and the minus-end directed Kinesin-14 HSET rescued chromosome alignment relative to hNuf2 knockdown alone. This alignment required the plus-end directed kinesin CENP-E, which mediated lateral binding of kinetochores to a subclass of spindle microtubule bundles. These bundled microtubules likely arise from the changes in spindle organization in cells lacking HSET. While chromosomes were still able to align in cells lacking K-fibers, the congressed chromosomes were not stably maintained at the metaphase plate. Our data challenge the widely accepted model that K-fibers contribute to chromosome congression by showing that neither chromosome bi-orientation nor a stable K-fiber

attachment is needed for chromosome congression. Instead our data support the idea that a major function of the K-fiber is to maintain chromosome alignment and highlight the importance of proper organization and structure of the spindle.

1506

Brownian Search and Strain-Dependent Catch Mechanism for Directional Motion of Myosin-VI.

T. Yanagida, M. Iwaki; Graduate School of Frontier Biosciences, Osaka University, Suita, Japan

Myosin-VI is a two-headed structure when bound to endocytic vesicles in cells that processively moves along actin helical pitches in a hand-over-hand fashion. The movement begins when ATP binds to the rear head causing the head to release from actin. A key question is how does the released head search for and bind to the forward actin target. The detached head undergoes Brownian motion back and forth along an actin filament, repeating rapid attachment and detachment with actin (weak-binding). This head is expected to endure backward strain near the forward target region at a moment when the head attaches to actin. Thus we examined the possibility that the backward strain might accelerate the transition from weak to steady strong binding. We applied forward and backward strains to a single-headed myosin-VI in the weak binding state tethered to an optically-trapped bead by moving the bead back and forth at various speeds, which resulted in short- and long-lived attachments. Short (500 μs)-lived attachments were independent of the direction of strain, suggesting weak-binding. Long-lived attachments greatly accelerated only when moved backward at sufficiently high speeds and when the dwell time (100 ms) was consistent with that of strong binding. We propose that the myosin-VI head searches for the forward actin target by Brownian motion and binds to it in a strain-dependent manner. This mechanism could be applied to other molecular motors, like kinesin and dynein, which lack lever arms rigid enough to tilt in a manner that biases the Brownian motion forward.

Minisymposium 17: 3-D Electron Microscopy (1507 – 1512)

1507

Ribosome-Induced Changes in EF-Tu Conformation: the Mechanism of GTP Hydrolysis.

E. Villa[1,2], J. Sengupta[3], L. Trabuco[1,2], J. LeBarron[3], W. T. Baxter[3], T. R. Shaikh[3], R. A. Grassucci[4], P. Nissen[5], M. Ehrenberg[6], K. Schulten[1,7], J. Frank[4,8]; [1]Beckman Institute, University of Illinois at Urbana-Champaign, Urbana, IL, [2]Center for Biophysics and Computational Biology, University of Illinois at Urbana-Champaign, Urbana, IL, [3]Wadsworth Center, Albany, NY, [4]HHMI, Biological Sciences, Columbia University, New York, NY, [5]Molecular Biology, University of Aarhus, Aarhus, Denmark, [6]Cell and Molecular Biology, Biomedical Center, Uppsala, Sweden, [7]Physics, University of Illinois at Urbana-Champaign, Urbana, IL, [8]Biochemistry and Molecular Biophysics, Columbia University, New York, NY

During translation, EF-Tu presents the aminoacyl-tRNA to the mRNA-programmed ribosome. Ribosome-induced GTPase activity of EF-Tu triggers conformational changes of the factor, that play a pivotal role in the selection and delivery of the cognate tRNA. A 6.7-Å resolution cryo-EM map of the aminoacyl-tRNA○EF-Tu○GDP○kir ternary complex-bound <it> E. coli </it> 70S ribosome reveals key conformational changes in the conserved switch regions of EF-Tu, which form a pocket for the γ-phosphate of the nucleotide. Using a novel molecular dynamics-based flexible fitting method, an atomic model based on the EM data has been obtained. The model allows the characterization of the binding interaction between the factor and the ribosome at unprecedented detail, revealing the conformational changes in the conserved GTPase switch regions of EF-Tu that trigger hydrolysis of GTP, along with key interactions, including those between the sarcin-ricin loop and the P-loop of EF-Tu, and between the effector loop of EF-Tu and a conserved region of the 16S rRNA. The structural evidence supports the hydrophobic gate model, i.e., the suggestion that interaction with the ribosome opens a gate formed by residues Ile60 and Val20 which, when open, allows His84 to act as a general base activating an active-site nucleophilic water molecule, thus triggering hydrolysis.

1508

Structural and Functional Significance of the N-Terminus of Connexin26 Gap Junction Channels.

A. Oshima[2], K. Tani[2], M. Toloue[2], Y. Hiroaki[2], A. Smock[1], B. Nicholson[3], G. Sosinsky[1], Y. Fujiyoshi[2]; [1]National Center for Microscopy and Imaging Research, Neurosciences, University of California, San Diego, La Jolla, CA, [2]Biophysics, Kyoto University, Kyoto, Japan, [3]Biochemistry, University of Texas Health Sciences Center, San Antonio, TX

Investigations of connexin channels have used different and often complementary experimental approaches to elucidate the gating mechanism of these intercellular conduits. No consensus gating model explaining the different phenomena of voltage gating, chemical gating and signal transduction has been formulated that is consistent with all mutagenesis, dye permeation, electrophysiology and electron microscopy results. Recent studies have shown that the N-terminus is an important regulatory component in gating, however the resolution of current EM crystal structures is insufficient to assign this domain to specific protein stretches in the channel structure and deduce a mechanism for pore closure. We made several N-terminal deletion constructs of Connexin26 (Cx26) to assess channel structure/function. Shorter deletions (del2-7 and del2-8) formed gap junction structures when expressed in mammalian cells, while longer deletions (del2-10 and del2-11) failed to form gap junctions, or did so less efficiently. For our structure analysis, a full-length Cx26 (Cx26M34A) and an N-terminal deletion construct (Cx26M34Adel2-7) were over-expressed in Sf9 insect cells. Purified proteins were reconstituted into lipid bilayers that formed 2D crystals. A 10Å 3D map revealed that crystals obtained from both constructs contained three lipid bilayers with the channels forming a p22121 lattice. The Cx26M34A structure showed a prominent density ("plug"), which resides in each hemichannel pore and contacts the innermost helices of surrounding subunits at the bottom of the vestibule. This was consistent with the lack of dye transfer through Cx26M34A channels and their dramatically reduced conductance when expressed in Xenopus oocytes. Since the voltage gating characteristics of the residual conductance were normal, it suggested that this plug was not a voltage gate. The Cx26M34Adel2-7 3D structure contained a reduced plug and a partially reduced density in the cytoplasmic domain that bridges the adjacent 4-helix bundles. This plug may contribute to the closure of Cx26M34A channels; however, it is likely to represent only one of multiple gating configurations, as gap junctions composed of Cx26M34Adel2-7 still showed no conductance.

1509

The Structure of Dynamin Family Members.

J. Mears, S. Fang, P. Ray, J. Heymann, J. Hinshaw; LCBB, NIH, Bethesda, MD

The dynamin family of proteins play a role in membrane fission events throughout eukaryotic cells. Dynamin is involved in the final stages of fission during endocytosis and Drp1 (dynamin-related protein 1) is necessary for mitochondrial division. During endocytosis, dynamin is believed to wrap around the necks of coated pits and facilitate vesiculation. In support of this model, we have previously shown by electron microscopy that purified dynamin self-assembles into spirals (50 nm diameter) and readily forms dynamin-lipid tubes, which constrict and fragment upon addition of GTP. The three-dimensional structures of dynamin in the constricted and non-constricted states were solved by cryo-electron microscopy methods, and using a rigid-body Monte Carlo algorithm, the crystal structures of the GTPase and pleckstrin homology domains were fit to the cryo-EM densities. The GTPase domain is placed at the periphery of the helical array while the Pleckstrin Homology domain is in a position that allows for interactions with lipid headgroups. The placement of the crystal structures into the cryo-EM densities revealed a twisting motion of the GTPase, middle and GTPase-effector domains, which suggests a corkscrew model for dynamin constriction. To determine if a common mechanism of action exists among the dynamin family members, we examined the structure and function of the Drp1 homologue in yeast, Dnm1. In collaboration with Dr. Jodi Nunnari (UC Davis) we have shown that Dnm1 assembles into large spirals with a diameter of 100 nm, the same diameter as the observed mitochondrial constriction sites seen in vivo. Dnm1 also assembles onto lipid and forms Dnm1 decorated tubes that constrict significantly upon GTP addition. A preliminary 3D map of Dnm1-lipid tubes reveals a similar architecture to dynamin, however, the Dnm1 map lacks a strong inner radial density near the lipid bilayer. This correlates well with the absence of a Pleckstrin Homology domain in Dnm1. These results suggest that dynamin family members share common mechanochemical properties that are uniquely tailored to fit their function.

1510

Spatial Architecture of Membrane Cytoskeleton and Spatial Specificity of Actin Binding Proteins Revealed by Immuno-Freeze Etching and Cryo-Microscopy.

J. Usukura[1], A. Yoshimura[1], T. Watanabe[2], K. Kaibuchi[3]; [1]EcoTopia Science Institute, Nagoya University, Nagoya, Japan, [2]Institute of Advanced Research, Nagoya University, Nagoya, Japan, [3]Department of Neuropharmacology, Graduate School of Medicine, Nagoya University, Nagoya, Japan

Three-dimensional architecture of membrane undercoat (membrane cytoskeleton) was investigated by freeze deep etched replica and cryo-electron microscopy combined with immuno-labeling. The membrane undercoat consisted of abundant actin filaments, intermediate filaments and microtubules together with their associated proteins independently from cytoplasmic cytoskeleton. Cryo-electron microscopy using native and hydrated specimen without any treatment revealed microtubules as well as actin filaments extending with wavy curvature. Such filamentous images, in particular microtubules, were quite different from the fixed specimen showing linearity. On the other hand, careful observation of freeze-deep-etching replica let us classify the cytoskeletal actin filaments into three types according to the spatial distribution. The first type is the actin filaments adhered firmly with the membrane surface in all extending areas. The second type is the ones extending parallel to the membrane surface while contacting salutatory with the membrane at several points. The third type is so called stress fibers that did not contact with the membrane except at their proximal ends. We also examined the localization of IQGAP1, actin-binding protein, that is involved in the formation of lamella-podia and one of effectors of Cdc 42. Interestingly, IQGAP1 was found predominantly on the actin filaments contacting salutatory with the membrane in cortical areas (type2 actin filaments), but not on stress fiber. Thus, localization of IQGAP1 showed clearly spatial specificity. Similar spatial specificity of actin binding proteins such as N WASP , VASP and Sra1 was detected as well. Cryo-electron microscopy using unroofed native samples also revealed first time smooth ER network as ubiquitous structure surrounding the cell that is located just beneath the plasma membrane.

1511

Cryo-Electron Tomography of Isolated Cytoskeletons of Giardia Intestinalis.

A. Hoenger[1], S. Dawson[2], C. L. Schwartz[1]; [1]MCD-Biology, Univ. of Colorado, Boulder, CO, [2]Dept. of Microbiology, Univ. of California Davis, Davis, CA

Giardia intestinalis is a ubiquitous unicellular, flagellated intestinal parasite of mammals with a two-stage lifecycle. Giardiasis is caused by ingestion of the cyst form which then excysts in the duodenum into the trophozoite form that directly attaches to the intestinal epithelium. The trophozoite form has a highly specialized microtubule cytoskeleton (eight flagella, the median body and the ventral disc (VD). The mechanism of attachment is not fully understood, but evidence suggests the VD is a key component. The VD consists of a counterclockwise array of microtubules (MT) that originate from electron dense bands anterior to the caudal and posterior-lateral basal bodies. The ventral surface of each MT is attached to the plasma membrane. On the dorsal side of each MT is a microribbon (MR) that is connected to neighboring MRs by cross-linkers. Previous studies of the Giardia cytoskeleton have focused on understanding the basic VD structure using low-resolution techniques. In our study, we are using cryo-electron tomography of isolated cytoskeletons to determine the structural-functional relationships between the various components of the VD. Detergent-extracted cytoskeletons were plunge-frozen on holey-carbon electron microscopy grids. These cytoskeleton preparations remain largely intact with the VD and all flagella present in most cases. The periphery of the VD was thin enough for cryo-tomography and reconstructions show excellent structure. Using a volume averaging software developed in our lab, we are able to obtain 3D volume averages of novel structures in the VD with a resolution approaching 3.4nm. We can clearly distinguish a 16nm repeat along the length of the MR. The MTs of the VD have protein complexes that are associated with 9 of the 13 protofilaments. These complexes are large, have an 8nm repeat, and can be at different angles (~48° and ~74°) to the long axis of the MT, indicating that the VD can be flexible in vitro. Our use of cryo-electron tomography in combination with 3D volume averaging is invaluable for allowing us to visualize the structural components of the VD and gain information on their roles in giardial adherence to the host intestinal epithelium.

1512

Visualizing Flock House Virus Infection in Drosophila Cells with Fluorescence and 3D Electron Microscopy.

J. Lanman[1], J. Crum[2], G. Gaietta[2], T. J. Deerinck[2], A. Schneemann[1], G. Sosinsky[2,3], M. H. Ellisman[2,3], J. E. Johnson[1]; [1]Molecular Biology, The Scripps Research Institute, La Jolla, CA, [2]National Center for Microscopy and Imaging Research, Center for Research on Biological Structures, La Jolla, CA, [3]Department of Neurosciences, University of California, La Jolla, CA

Virus assembly occurs in a complex cellular environment and is dependent upon both viral and cellular components being properly correlated in time and space. The simplicity of the Flock House virus (FHV) capsid and the extensive structural, biochemical, and genetic characterization of the virus make it an excellent system for studying in vivo virus assembly. Fluorescent microscopy and electron microscope tomography (EMT) were used to investigate the spatial organization of FHV assembly in the cell. The virus particles consist of a coat protein and two viral RNA segments. RNA 1 encodes for the RNA dependent RNA polymerase (RdRp), which forms spherules on the outer mitochondrial membrane. These spherules are the location for RNA replication. Additionally, the mitochondria are convoluted in on themselves to form large chambers lined with these spherules. RNA2 encodes the coat protein, which forms the T=3 icosahedral viral capsid. Following assembly, the virus particles form paracrystaline arrays in the cytoplasmic space. Using new sample preparation approaches and tomographic imaging technologies we determined the three-dimensional structure of the RdRp protein complex inside these spherules. In these tomograms, the RdRp complex was observed associated with small fibrils, and the paracrystaline arrays were associated with large bundles of actin fibrils (~200 to 300 nm). Our current model is that the virus coat protein is synthesized in close vicinity to the modified mitochondria, binds to the actin fibrils (potentially facilitating assembly) and is then trafficked on bundles of actin fibrils to a central location to form viral arrays. These extensive changes in the cell to form elaborate viral factories indicate that virus assembly is a highly ordered process.

Minisymposium 18: Apoptosis (1513 – 1518)

1513

Regulation of Anoikis by Ankyrin Complexes.

S. Frisch, S. Kumar; West Virginia University, Morgantown, WV

Detachment of epithelial cells from the extracellular matrix or attachment to an inappropriate matrix engages an apoptotic response known as anoikis. While mechanisms of anoikis regulation by integrins have been addressed in part, the role of E-cadherin-mediated interactions in sensitizing epithelial cells to anoikis is documented but not yet understood. Herein, we report that the E-cadherin interacting cytoskeletal protein ankyrin-G forms complexes with apoptosis-regulatory proteins so as to control anoikis. Ankyrin-G dissociates from the cytoskeleton rapidly following cell-matrix detachment, affecting its function. This protein has two domains that interact with potential apoptosis-regulatory proteins, a Death domain and ZU-5 domain. The TNFR1-interacting death domain protein RIP1 (RIPK1) interacted with the death domain of ankyrin-G. The Neurotrophin Receptor-interacting Melanoma Antigen (NRAGE/MAGE-D1) interacted with the ZU-5 domain. Overexpressed ankyrin induced apoptosis in a RIP1-dependent manner. By contrast, NRAGE was an anoikis suppressor. A reciprocal control of ankyrin and NRAGE localization, with functional consequences, was observed. Over-expressed NRAGE compromised the lateral membrane localization of ankyrin-G, causing the disorganization of adherens junctions containing E-cadherin. Reciprocally, ankyrin-G sequestered NRAGE in the cytoplasm. E-cadherin depletion caused the loss of ankyrin-G protein, an increased nuclear localization of NRAGE, and resistance to anoikis. Consistent with its role as an anoikis suppressor, NRAGE was over-expressed in a significant fraction of breast and lung tumors. This study reveals important roles for ankyrin-G interactions with E-cadherin, NRAGE and RIP1 in the regulation of anoikis.

1514

The Ubiquitin Ligase Itch Protects Cells from Apoptosis by Promoting tBID Ubiquitylation and Proteasomal Degradation.

B. A. Azakir, G. Desrochers, A. Angers; Biology, University of Montreal, Montréal, QC, Canada

Itch is an ubiquitin ligase of the Nedd4 family whose substrate binding and enzymatic activity is stimulated by JNK-mediated phosphorylation. Using a candidate approach, we identified tBid as a substrate of Itch. tBid appears in cells after activation of caspase 8 by pro-apoptotic stimuli. Caspase 8 cleaves the inactive protein Bid to produce the proapoptotic C-terminal fragment, tBid. tBid promotes apoptosis by recruiting Bax or Bak to mitochondria which induce cytochrome c liberation. Full length Bid is not recognized nor ubiquitylated by Itch, but tBid is. Overexpression of Itch significantly reduced the amount of tBid accumulating in cells after overexpression of Bid. Consistently, transfection of Itch in HEK-293T cells significantly increased cell survival and reduced caspase 3 activity, whereas reducing Itch expression with RNAi reduced cell survival and increased caspase 3 activity. Exogenous tBid expression induces massive cell apoptosis. Itch expression was able to rescue cells from tBid-induced apoptosis. Downregulation of Itch had the oposite effect, resulting in increased caspase activation and cell death. Itch expression was also able to rescue HEK-293T cells from TRAIL-induced apoptosis. It has been reported earlier that EGF treatment also protected cells from ligand-induced apoptosis through a mechanism that seemed to involve Bid or tBid expression. We reported that treatment of HEK-293T cells with EGF stimulates Itch activity through JNK activation. Consistently, we here show that downregulation of Itch partly abolishes the ability of EGF to protect HEK-293T cells from TRAIL-induced apoptosis. These results show that Itch expression and activation can protect cells form apoptosis, and that the antiapoptotic effects of EGF may at least in part be attributed to Itch activation. Our findings identify Itch as a key molecule between EGF signaling and resistance to apoptosis, providing further details on how EGF signaling and proteasome inhibitors can both contribute to induction of apoptosis and treatment of cancer.

1515

Bax Enhances Mitochondrial Outer Membrane Permeabilization (MOMP) by Enlarging Ceramide Channels.

V. Ganesan, M. Perera, M. Colombini; Biology, University of Maryland, College Park, MD

Bax is a pivotal pro-apoptotic Bcl-2 family protein that localizes to mitochondrial outer membrane (MOM) during apoptosis and causes MOM permeabilization (MOMP). Earlier studies have demonstrated that upon an apoptotic stimulus, intracellular production of ceramide, a bioactive pro-apoptotic lipid, often greatly increases including in the MOM. Elevation of ceramide in the MOM is sufficient to cause MOMP without requiring any Bcl-2 family proteins. Moreover ceramide induced MOMP is reversible/preventable by the anti-apoptotic protein, Bcl-xL. Using isolated rat liver mitochondria and a dynamic cytochrome c accessibility assay, we show that Bax induces MOMP by enlarging ceramide channels. While ceramide forms channels independently of Bax, their permeabilization is enhanced by the addition of less than 5nM oligomeric Bax. As much as 50nM oligomeric Bax alone does not result in any MOMP. The Bax mediated channel enlargement occurs with an apparent affinity that increases with an increase in ceramide-induced MOMP. Perhaps this explains the underlying mechanism by which Bax enhances ceramide-induced MOMP. Bax also causes ceramide channel enlargement in more simplified systems such as yeast mitochondria, which lack Bcl-2 family proteins, as well in planar phospholipid membranes, which is a defined, protein free, system. By contrast, monomeric Bax has no effect on ceramide channels in the aforementioned systems. The Bax inhibitor, Bci2 [Bruno Antonsson], prevents Bax mediated channel enlargement but does not affect permeabilization induced by ceramide alone. Thus both pro- and anti-apoptotic proteins regulate ceramide channels consistent with ceramide channels being the pathway by which proteins are released by mitochondria early in apoptosis. (Supported by NSF grant: MCB-0641208.

1516

BubR1 Is a Potential Key Regulator of Mitochondrial Apoptotic Cell Death.

M. Kim, C. Yoon, J. Byun, S. Lee; Hanyang University, Seoul, South Korea

Mitotic checkpoint protein BubR1 plays a dual role in the maintenance of genomic integrity and the regulation of apoptotic cell death. We show here that BubR1 is necessary for regulating mitochondrial cell death in response to anticancer agents. BubR1 directly activated the proapoptotic Bcl-2 family protein Bax and Bak to permeabilize mitochondrial membrane and engage the apoptotic program in response to anticancer agents. The conformational activation of Bax and Bak by BubR1 occurred with its direct interaction with both antiapoptotic Bcl-2 and Bcl-XL proteins. BubR1 also released proapoptotic multidomain proteins and BH3-only proteins that were sequestered by Bcl-2 or Bcl-XL. We propose that when cells are exposed to anticancer agents, BubR1 can function similarly to the BH-3 only subset of proapoptotic Bcl-2 family proteins to activate Bax and Bak and trigger mitochondrial apoptotic cell death, suggesting that BubR1 is a potential key regulator of mitochondrial apoptotic cell death.

1517

Cross-Talk between the Cell Cycle Machinery and Apoptosis.

S. Kornbluth; Dept. of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC

In response to chemotherapeutics and other cytotoxic agents, cells initiate the intrinsic pathway of apoptosis, leading to mitochondrial cytochrome c release. Once released, cytochrome c binds to Apaf-1, which oligomerizes and recruits and activates the initiator caspase, caspase 9 in a structure known as the apoptosome. Using an apoptotic in vitro reconstitution system derived from Xenopus eggs, we have found that both mitochondrial cytochrome c release and the apoptosome are inhibited in mitosis. The pre-mitochondrial inhibition appears to stem from modification of caspase 2, an initiator caspase that can function upstream of mitochondria to induce cytochrome c release. Although it has been reported that phosphorylation of caspase 9 by Cdc2/Cyclin B kinase is responsible for apoptosome inhibition in mitosis, we find that it is the interaction between Apaf-1 and cytochrome c, rather than a modification of caspase 9, that is critical for this inhibition. We are currently identifying post-translational modifications of Apaf-1 that may account for this effect. We have also recapitulated these results in tissue culture systems and speculate that mitotic inhibition of apoptosis in somatic cells prevents the cell death that would otherwise occur when cells round up and lose contact with neighboring cells at mitosis. We are also investigating the possibility that caspase 2 modification is important for regulating mitotic catastrophe. In studying the interface between apoptosis and the cell cycle, we have also characterized a known apoptotic regulator, Aven, as a key component of the DNA damage checkpoint that prevents G2/M progression in the presence of damaged DNA. Our data indicate that Aven promotes activation of the ATM kinase. This places Aven with a number of other apotptotic regulators (eg. Bid, Apaf-1) that appear to function in both apoptosis and cell cycle checkpoint regulation.

1518

An Engulfment Pathway in Sertoli Cells that Regulates Apoptotic Germ Cell Removal and Spermatogenesis.

M. R. Elliott[1], J. J. Lysiak[3], D. J. Park[2,1], R. I. Woodson[3], A. C. Tosello-Trampont[1], K. S. Ravichandran[1,2]; [1]Carter Immunology Center, University of Virginia, Charlottesville, VA, [2]Microbiology, University of Virginia, Charlottesville, VA, [3]Urology, University of Virginia, Charlottesville, VA

Spermatogenesis is a complex process where the proliferation/differentiation of germ cells is intimately tied to their apoptosis. Approximately 50% of germ cells undergo apoptosis during differentiation, yet few apoptotic cells are observed under normal conditions. The Sertoli cell is the predominant somatic cell in the seminiferous tubules and is thought to mediate the rapid and efficient phagocytic removal of apoptotic germ cells during spermatogenesis. However, the signaling pathways in Sertoli cells important for this engulfment are not known. The ELMO/Dock/Rac signaling module has been shown to promote phagocytosis of apoptotic cells in macrophages and other phagocytes. Here we identify the ELMO/Dock/Rac module as a key pathway in Sertoli cell-mediated engulfment of apoptotic germ cells in vitro and in vivo. Using in vitro and biochemical approaches we found that ELMO1, Dock180, and Rac1 are expressed in Sertoli cells and function to promote engulfment of apoptotic germ cells. In vivo studies revealed that mice lacking ELMO1 display severely disrupted organization of the seminiferous tubules, characterized by abnormal germ cell differentiation, sharply decreased numbers of mature sperm, and significantly increased levels of uncleared apoptotic germ cells. Moreover, Sertoli cells isolated from ELMO1-deficient mice were intrinsically impaired in their ability to phagocytose apoptotic targets. In addition, through the use of a decoy peptide, we found that BAI1, a recently identified apoptotic cell receptor upstream of ELMO/Dock/Rac, is also important for the removal of apoptotic germ cells in vivo. These results identify for the first time an engulfment signaling pathway in Sertoli cells that regulates the clearance of apoptotic germ cells and spermatogenesis.

Minisymposium 19: Cell Polarity and Epithelial Morphogenesis (1519 – 1524)

1519

The Drosophila Crumbs-Stardust Protein Complex and Its Role in Epithelial Polarity and Retinal Morphogenesis.

E. Knust; Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

A polarised phenotype reflects the ability of a cell to establish functionally and spatially separated compartments. There is accumulating evidence that multiprotein complexes are instrumental to compartmentalise the plasma membrane and the underlying cytocortex. The Drosophila MAGUK-protein Stardust organises a protein complex apical to the zonula adherens of epithelial and photoreceptor cells by recruiting the transmembrane protein Crumbs and the PDZ-domain containing proteins DPATJ and DLin-7. One of the objectives of our studies is to understand how the various domains of Stardust and Crumbs contribute to the function of the complex. For this, we expressed mutant versions of the two proteins in either wild-type or mutant background and tested for their ability i) to concentrate apically, ii) to localise the other members of the complex, and iii) to rescue the mutant phenotype in the embryo or the photoreceptor cell. The results show that in early pupal photoreceptors, only Crumbs is necessary for the organisation of the complex. In the later pupae and the adult eye, Crumbs and Stardust depend on each other in their localisation. These results led us to conclude that more complex mechanisms regulate the Crumbs-Stardust complex in the adult photoreceptor cells.

1520

A Netrin Ligand Directly Stimulates Cell Invasion through Basement Membranes In Vivo.

J. Ziel, D. R. Sherwood; Department of Biology, Duke University, Durham, NC

The ability of invasive cells to penetrate and migrate through basement membranes is critical during development and contributes to tumor progression and metastasis. Despite obvious importance, neither the intracellular pathways that promote cell-invasive behavior, nor the extracellular cues that stimulate invasion are well understood *In Vivo*. By analyzing the genetic control of a developmentally-regulated cell invasion event in *C. elegans*, anchor cell (AC) invasion into the vulval epithelium, we have identified a novel role for the UNC-6/Netrin signaling pathway in promoting basement membrane transmigration. Through phenotypic analysis of null mutants and mosaic rescue experiments we show that UNC-6/Netrin, secreted from ventral cord neurons, acts directly on the AC, where it polarizes its receptor, UNC-40/DCC, to the basal plasma membrane. Using fluorescently tagged proteins and live-cell microscopy we show that polarized UNC-40/DCC marks a specialized invasive membrane domain containing F-Actin, the netrin signaling effectors UNC-34/Enabled, MIG-2/Rac and CED-10/Rac, as well as the phospholipid phosphatidylinositol 4,5-bisphosphate. At the invasive membrane, we show that each of these factors contributes to AC invasion and basement membrane removal. In addition we show that UNC-6-mediated polarization of the AC's invasive cell membrane is critical for normal response to a second cue emanating from the vulval cells, which stimulates invasion at the appropriate time. Because netrin signaling has been shown to regulate several developmental events that involve migrations through basement membranes (e.g. lung, pancreatic, and blood vessel development) this novel function for netrin signaling may be evolutionarily conserved. Moreover, our results highlight the intricate circuitry guiding regulated-cell invasions *In Vivo* and illustrate how multiple pathways maybe co-opted by metastatic cells during tumor progression.

1521

Epithelial Stem Cells of the Drosophila Ovary Utilize Basolateral Junctions to Maintain a "Dynamic Niche" and to Suppress Tumorgenesis.

T. Nystul, A. Spradling; Embryology, Carnegie Institution/HHMI, Baltimore, MD

The polarized follicular epithelium of Drosophila ovarian follicles is continuously generated by exactly two follicle stem cells (FSCs) which lie singly within niches on opposite sides of each ovariole (Nystul and Spradling, 2007). Unlike the germline stem cells, which are permanently held in place by a fixed stromal cell niche, FSCs contact only transient cell populations and maintain a position that is fixed only relative to their neighbors. Despite this, FSC niches are maintained throughout adult life, even as individual FSCs are regularly lost and replaced by competition for niche occupancy. The signals and microenvironment that maintain FSCs, which we term a "dynamic" niche, are likely to depend on transient cell-cell contacts and signals with nearby epithelial cells and extracellular matrices. Basolateral junctions are likely to be important because they interconnect follicular epithelial cells and medidate structural organization, signaling and growth control (as evidenced by tumor suppression). In a genetic mosaic screen for mutations that affect the rate of FSC replacement, we identified mutations in two genes which confer a "hyper-competitive" phenotype. Interestingly, both mutants have additional phenotypes that are known to be associated with basolateral junction defects. Follicle cells that are homozygous for either mutation exhibit cell autonomous loss of polarity, hyperplasia and metastasis. Larvae that are heterozygous for both mutations display a "lethal giant larvae" phenotype, suggesting that they function in a common pathway. We have analyzed whether these genes act directly on basolateral junctions or on downstream signals, and how these pathways affect stem cell replacement. These studies will advance understanding of "dynamic" niches and help elucidate the connection between stem cells, cell adhesion and cancer. Nystul, T. and Spradling, A.C. (2007). An epithelial niche in the Drosophila ovary undergoes long range stem cell replacement. Cell Stem Cell 1, 277-285

1522

Dissecting the Role of TBC1d19 in Planar Cell Polarity (PCP) Signaling in Vertebrates.

P. C. Rida, R. Schlisner, K. Radde-Gallwitz, P. Chen; Cell Biology, Emory University, Atlanta, GA

Planar cell polarity (PCP) refers to coordinated polarization of all the cells in a cell sheet. PCP is required for polarized cellular protrusive activities that drive convergent extension (CE) in mesenchymal tissues during gastrulation, and for establishing precise orientation of sensory hair cells in the inner ear. PCP in vertebrates is regulated by a signaling cascade comprising long-range directional cues, reception and translation of vectorial information via asymmetric segregation of membrane-bound protein complexes and positioning/configuration of the primary cilia/centrioles, and morphological polarization resulting from the concerted effect of membrane-bound PCP protein complexes and

centrioles on cytoskeleton. However, the signal transduction from membrane-bound PCP complexes and centrioles to the cytoskeleton remains a molecular black box. We performed a yeast two-hybrid screen with the C-terminal cytoplasmic domain of a transmembrane core PCP protein (Vangl2) from mouse to identify candidate genes that may function as the missing linkers in this transduction process. One of the genes identified is TBC1d19, a putative RabGAP protein containing a conserved TBC domain. In situ hybridization shows that the expression of TBC1d19 is similar to that of Vangl2 in gastrulating zebrafish embryos and in vertebrate inner ear sensory epithelia. Through morpholino-mediated knockdown experiments in zebrafish, we have been able to detect a genetic interaction between Vangl2 and TBC1d19 in the regulation of CE. TBC1d19 morphants show PCP defects in the inner ear and impaired Kupffer's vesicle morphogenesis. Overexpression of TBC1d19 in zebrafish also causes defects in CE and inner ear morphogenesis. Furthermore, TBC1d19 localizes to the centrosome and to the midbody in ciliated mIMCD3 cells, implicating a potential association of TBC1d19 with microtubule cytoskeleton and centrioles for PCP signaling. Detailed characterization of TBC1d19's intriguing role in PCP signaling is currently underway.

1523

The Scribble/Dlg/Lgl Polarity Module in the Control of Mammalian Development and Cancer.

L. E. Dow, N. Godde, I. Elsum, R. Galea, L. Smith, A. Ogden, S. Russell, H. Richardson, P. O. Humbert; Research Division, Peter MacCallum Cancer Centre, Melbourne, VIC, Australia

Genetic screens in Drosophila have identified scribble, discs large (dlg) and lethal giant larvae (lgl) as key epithelial polarity regulators with mutation in any of these genes resulting in loss of polarity, overproliferation and multilayering of epithelial cells leading to 3D-tumourous overgrowth, and in the presence of activated Ras, invasion and metastasis. Evidence from cancer patients suggests that Scribble, Dlg and Lgl could act as a tumour suppressor in some epithelial cancers with low levels and/or mislocalization of mammalian Scribble, Dlg and Lgl homologues correlating with increased tumour invasiveness and malignancy. The actual role of mammalian Scribble/Dlg/Lgl complex in development and tumourgenesis however remains poorly understood. Here, we have used a combination of in vivo animal analysis, biochemical studies and RNAi screening to uncover the functional role of Scribble complex in mammalian cells. Using existing mouse mutant alleles for Dlg1 and Scribble and 3D cell culture models, our original analysis demonstrated that mammalian Scribble and Dlg act in concert to promote or inhibit migration dependent on the cellular context. To bypass the neonatal lethality of existing mouse mutants, we have now generated a novel conditional allele of Scribble in the mouse. We show that Scribble is required for mammary development in vivo and will discuss how Scribble impacts on this process in view of our recent demonstration that Scribble can regulate the Ras-Raf-MAPK pathway both in mammals and Drosophila. In addition, we have undertaken focussed and genome wide shRNA screens to characterize the functional network that underlies Scribble function. Initial results from these screens have revealed novel functional interactions between various mammalian polarity complexes as well as key signalling components. We propose that Scribble, Dlg and other associated polarity proteins are signalling regulators involved in a new pathway controlling epithelial tumour progression in mammals.

1524

An Unexpected Role for the Par3 Polarity Protein in Mammary Ductal Morphogenesis and Progenitor Cell Differentiation.

I. Macara, L. McCaffrey; Center for Cell Signaling, Univ. of Virginia, Charlottesville, VA

Mammalian polarity protein functions have been studied predominantly in cell culture systems. It is important, however, to determine if there is a correspondence between functions revealed in these systems and those that occur in vivo. To address this issue, we have developed a lentiviral system to manipulate gene expression in murine mammary stem cells. The effects of such manipulation can be determined after transplanting the stem cells into a host mouse and permitting regeneration of a mammary gland from the cells. Unexpectedly, silencing of Par3 disrupted normal ductal outgrowth. The glands were characterized by altered rates of proliferation and apoptisis, and an expanded progenitor pool. Expression of a full-length human Par3 could rescue normal morphogenesis, but a mutant that cannot bind atypical PKC was unable to rescue. These results demonstrate the power of using lentiviral transduction of stem cells together with organ regeneration in host mice, and reveal a novel function for Par3 in the regulation of mammary progenitor and epithelial morphogenesis.

Minisymposium 20: Cellular Response to Infectious Agents (1525 – 1530)

1525

Analysis of the Interaction of HIV with Female Genital Tract Tissue as a Model to Understand Sexual Transmission.

T. Hope[1], A. Trull[1], S. McCoombe[1], M. McRaven[1], R. S. Veazey[2]; [1]Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL, [2]Division of Comparative Pathology, Tulane University, Covington, LA

Sexual transmission accounts for over 80% of all HIV infections. To establish infection HIV must reach its target cells of infection, which include CD4 positive T cells, macrophages, Langerhans cells and dendritic cells that are protected by mucosal epithelial barriers. Previous attempts to observe HIV interactions in tissue explant and primate models have been hindered by high background fluorescence associated with tissue. Therefore, to study the interaction of single virions with genital tissues, we developed a new method utilizing photoactivatable GFP (PA-GFP) labeled HIV. Using this system, tissue autofluorescence is defined, the sample is photoactivated and the newly detected signal is labeled HIV. To study initial HIV interactions with the mucosal barrier we utilized tissue obtained from hysterectomies and a rhesus macaque sexual transmission model. Following inoculation, tissue samples were frozen, sectioned and stained, enabling observation of tissue structure, HIV target cells and PA-GFP labeled virions. This analysis revealed that HIV penetrates the superficial squamous epithelia of the female genital tract in both the human explant and living macaque models to depths of up to 50 microns within 4 hours. Penetration to this depth is sufficient to allow interaction with resident HIV target cells. Interestingly, the penetrating virions are primarily observed between differentiated squamous epithelial cells revealing that the particles are able to move in the interstitial spaces between the cells. Co-application of fluid phase markers reveals that the virus primarily enters the tissue in places where water also enters the tissue. Further analysis of the expression of proteins involved in forming cell-cell junctions revealed that they are deficient in the areas of virus entry. Together these results suggest that HIV can enter the tissue by diffusion into areas of the squamous epithelium that are degenerating as part of the normal turnover

of the squamous epithelium. This is an unexpected and important result because it is generally believed that the squamous epithelium of the female genital tract is an efficient barrier to viral penetration.

1526

Conjugated Action of Two Species-Specific Invasion Proteins for Fetoplacental Listeriosis.

M. Lecuit; [1]Department of Cell Biology and Infection, Institut Pasteur, Paris, France, [2]Department of Infection and Epidemiology, Institut Pasteur, Paris, France, [3]Hôpital Necker-Enfants malades, Université Paris Descartes, Paris, France

The ability to cross host barriers is an essential virulence determinant of invasive microbial pathogens. Listeria monocytogenes is a model microorganism that crosses the human intestinal and placental barriers, and causes severe maternofetal infections by an unknown mechanism. A number of studies have helped to characterize the bacterial invasion proteins InlA and InlB. However, their respective species specificity has complicated investigations on their in vivo role. Herein, we first describe two novel and complementary animal models for human listeriosis: the gerbil, a natural host for L. monocytogenes, and a knock-in mouse line ubiquitously expressing humanized E-cadherin. Using these two models, we uncover the essential and interdependent roles of InlA and InlB in fetoplacental listeriosis, and thereby decipher the molecular mechanism underlying the ability of a microbe to target and cross the placental barrier.

1527

Actin-based Motility of Baculovirus Plays a Key Role in Transport to the Nucleus and Cell Surface.

T. Ohkawa, L. E. Volkman, M. Welch; Molecular and Cellular Biology, University of California, Berkeley, CA

Autographa californica M nucleopolyhedrovirus (AcMNPV), the type-species of baculoviruses, enters cells by endocytosis, is released into the cytosol, and travels to the nucleus where it replicates. However, the mechanism of intracellular transport has remained undetermined. We previously showed that the AcMNPV protein p78/83, which is localized to one end of the nucleocapsid particle, is a nucleation promoting factor (NPF) that activates the host Arp2/3 complex to promote actin polymerization. We sought to determine whether p78/83 promotes actin assembly to drive actin-based motility, as is the case with other pathogen-encoded NPFs. By visualizing individual nucleocapsids labeled with RFP in cells expressing EGFP-actin using time lapse fluorescence microscopy, we found that AcMNPV nucleocapsids are indeed propelled by actin-based motility, resulting in the formation of actin comet tails that trail the moving virions. Motility involves the activity of p78/83, as a virus carrying a point mutation in p78/83 that reduced Arp2/3 complex activation also dramatically slowed actin-based motility. In a striking departure from what is seen with other pathogens, we observed that moving nucleocapsids collided with and stuck to the nuclear envelope, where actin accumulated in corkscrews radiating from the nuclear surface. Nucleocapsids eventually separated from these corkscrews and moved into the nucleus. Actin-based movement played a critical role in transit to the nucleus, as treatment of cells with drugs that interfere with actin dynamics delayed the onset of viral early gene expression and reduced the percentage of cells with detectable expression. In addition to its role in nuclear transit, we observed that actin-based movement propelled nucleocapsids into the tips of cell surface spikes, where virions accumulated following viral early gene expression. Accumulation of nucleocapsids at the cell surface may enable rapid spread between cells during infection. Thus baculoviruses appear to have evolved mechanisms for using actin-based motility to facilitate processes including intracytoplasmic transport, nuclear entry, and rapid spread during infection.

1528

Retroviral Assembly Is Directed Towards Sites of Cell-Cell Contact.

J. Jin[1,2], N. Sherer[1,2], W. Mothes[1,2]; [1]Microbial Pathogenesis, Yale School of Medicine, New Haven, CT, [2]Department of Cell Biology, Yale University School of Medicine, New Haven, CT

Retroviruses efficiently spread from cell to cell. Indeed, under conditions of cell-cell contact, HIV spreading is 100-10,000 fold more efficient as compared to cell free virus(Carr et al., 1999; Chen et al., 2007; Dimitrov et al., 1993; Hope, 2007; Sourisseau et al., 2007). This enhancement is believed to be the consequence of the re-direction of virus assembly and budding towards sites of cell-cell contact as well the directed transport of released particles from one cell to another(Igakura et al., 2003; Johnson and Huber, 2002; McDonald et al., 2003; Sherer et al., 2007; Sowinski et al., 2008). Applying 4D imaging to study this process we detect the assembly of individual murine leukemia virus particles at the plasma membrane. Single virus particles assemble on average within ~13 min, slightly slower as compared to the 8.5 min observed for HIV in HeLa cells(Jouvenet et al., 2008). Importantly, quantifying assembly sites at the plasma membrane and at sites of cell-cell contact revealed a strong enhancement of assembly in zones of contact. In these zones, viral particles form directly in contact with target cell receptor. Viruses then pinch off and move towards the target cell to spread the infection. In contrast, late domain mutant virus fails to completely pin off, is trapped in between cells and cannot infect neighboring cells. Our work directly demonstrates for the first time that virus assembly is directed towards sites of cell-cell contact thereby contributing to the efficient spreading of retroviruses.

1529

Inhibition of Host Cell Apoptosis by the Coxiella Burnetii Effector Protein Ankg.

C. Roy, A. Lührmann; Microbial Pathogenesis, Yale University, New Haven, CT

The obligate intracellular bacterium *Coxiella burnetii* is able to inhibit host cell apoptosis allowing this pathogen to survive and replicate intracellularly. The ability of *C. burnetii* to prevent host cell apoptosis is likely to involve a type IV secretion system called Dot/Icm, which delivers bacterial effector proteins into the host cell cytosol. *C. burnetii* proteins that are substrates of the Dot/Icm system were identified. Individual effectors were tested for the ability to prevent host cell apoptosis. A protein called AnkG was found to prevent staurosporine-induced apoptosis when expressed ectopically in mammalian cells. An amino-terminal region of AnkG was determined to be necessary and sufficient for this anti-apoptotic function. To better understand the molecular mechanism by which AnkG prevents apoptosis, host proteins that bind to AnkG were identified. A specific interaction between AnkG and the host protein p32 was demonstrated. Previous studies suggest that p32 is important for a splicing reaction that generates the pro-apoptotic BH3-only protein Bcl-Xs. Overproduction of p32 resulted in an increase in the spliced message encoding the pro-apoptotic protein Bcl-Xs. AnkG interfered with the p32-mediated splicing reaction that

produced Bcl-Xs. Thus, the *C. burnetii* effector protein AnkG is capable of inhibiting host cell apoptosis by a novel mechanism that involves binding to p32 and interfering with an alternative splicing reaction that results in production of Bcl-Xs.

1530

Cellular Responses to Pore-Forming Toxins.

F. van der Goot, M. Bischofberger, M. Gonzalez, B. Frêche; Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

Pore-forming toxins (PFTs) are secreted proteins that contribute to the virulence of a great variety of bacterial pathogens by perturbing plasma membrane permeability of the target cell. Recent work by our lab and others have highlighted that mammalian cells respond to pore-formation by activating numerous pathways, some of which contribute to prolonged cell survival. We previously found that the upstream event sensed by the cell and responsible for the activation of SREBP, the central regulator of lipid biosynthesis, was the decrease in cytoplasmic potassium levels. Our recent work has shown that numerous other pathways are activated in response to pore-formation. In particular, we found that the PFT-induced decrease in cellular potassium not only triggers the activation of SREBP, but also the activation of p38 and the phosphorylation of eukaryotic Initiation Factor 2α (eIF2α). In agreement, we found that protein translation was arrested. The arrest was transient and the recovery of protein synthesis was accompanied by a recovery of intracellular potassium levels. Moreover, recovery of intracellular potassium was both p38 and SREBP dependent. Based on these and previous findings we suggest that the defense pathways, p38 and SREBP, are activated in a potassium-dependent manner, as well as translational arrest through eIF2α. P38 and SREBP are then responsible for the recovery of the plasma membrane integrity leading to the prolonged survival observed in earlier publications. If time permits, additional cell response pathways to pore-formation will be discussed.

Minisymposium 21: Genomic Instability and Cancer (1531 – 1536)

1531

Position of Human Chromosomes Is Conserved in Mouse Nuclei Indicating a Species-independent Mechanism for Maintaining Genome Organization.

K. Sengupta, J. Camps, P. Mathews, L. Barenboim-Stapleton, Q. T. Nguyen, M. J. Difilippantonio, T. Ried; Section of Cancer Genomics, Genetics Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD

Chromosomes are non-randomly positioned in the interphase nucleus. Gene rich chromosomes (HSA19) are predominantly localized towards the nuclear center while gene poor chromosomes (HSA7 and 18) are proximal to the nuclear periphery. We previously showed that when human chromosomes were artificially introduced into diploid DLD-1 colon cancer cells by microcell mediated chromosome transfer (MMCT), both gene poor and gene rich chromosomes assumed a conserved position indistinguishable from their endogenous homologues. Introduction of an extra copy of chromosome 7 resulted in significantly increased average transcript levels (DLD-1+7), suggesting a concordance between transcriptional activity and a conserved chromosome position. The primary objective of this study was to understand if chromosome positioning is conserved in a species independent manner. We examined the position of fluorescently labeled human chromosomes in donor mouse-human hybrid cells used previously for MMCT by 3-Dimensional-fluorescence in situ hybridization (3D-FISH) and confocal imaging followed by 3D reconstructions and statistical analyses of radial distance measurements of chromosome territories from the center of the nucleus. Taken together, these analyses showed that human chromosomes assume conserved but divergent positions in foreign mouse nuclei equivalent to that of human nuclei. Endogenous mouse chromosomes however do not show such marked differences in position due to relatively smaller differences in gene density and size. Further, gene expression profiling of these hybrid cells using microarrays showed that the human chromosomes were transcriptionally active. Taken together, these analyses showed that a mouse nucleus is capable of partitioning human chromosomes at strikingly contrasting positions on the basis of their gene densities. Furthermore, a human chromosome retains its transcriptional activity even under these trans-species conditions, inseparable from its conserved nuclear position. These studies therefore demonstrate that a species independent mechanism operates to maintain a conserved, non-random chromosome positioning pattern in the nucleus, the molecular basis of which largely remains elusive.

1532

Gene Amplification through Loss of DNA Replication Control.

K. J. Finn[1], B. Green[2], S. Ricoult[1], J. Li[1]; [1]Department of Microbiology and Immunology, University of California San Francisco, San Francisco, CA, [2]Department of Molecular Biology & Genetics, Johns Hopkins University School of Medicine, Baltimore, MD

Gene amplification is frequently associated with carcinogenesis and used by cancers to evolve resistance to chemotherapy. The prevailing model to explain gene amplifications invokes repeated chromosomal cycles of breakage-fusion-bridge. However, this model cannot account for all examples of gene amplification observed in tumors, particularly those involving amplicons arranged in direct repeat. Our studies on the control of DNA replication in the budding yeast *Saccharomyces cerevisiae*, suggest that loss of replication control can be an extremely potent initiator of gene amplification. We show that limited and transient re-replication from a single replication origin induces the first step of gene amplification, segmental duplications, in 2-3% of re-replicating cells. This five order of magnitude increase beyond spontaneous duplication rates is specific to re-replication, as it is not observed with DNA damage or defective replication alone. Array-based comparative genomic hybridization and PCR analysis demonstrates that the duplicated segments can span at least 7 replicons, are bounded by repetitive elements (Ty retrotransposons), and arrayed in direct head-to-tail orientation at their endogenous locus. Sequence analysis across the Ty junctions and genetic analysis suggest that re-initiation at an origin greatly stimulates nonallelic homologous recombination between flanking Ty elements. Further analysis indicates that the recombination is mediated primarily through a single-stranded annealing mechanism. Our results thus resurrect the long neglected re-replication model for gene amplification and raises the possibility that cryptic re-replication contributes to gene amplification during human carcinogenesis.

1533

Chromosome Positioning and 3-D Folding Is Disrupted in Progenitor B Cell Lymphoma Nuclei.

K. J. Snow[1,2], L. Titus[1], S. Wright[1], K. D. Mills[1], L. S. Shopland[1]; [1]The Jackson Laboratory, Bar Harbor, ME, [2]Graduate School of Biomedical Science, Functional Genomics, University of Maine, Orono, ME

Translocations occur frequently in tumor cells and often deregulate oncogenes and tumor suppressors by disrupting a gene's coding or regulatory sequence. Although much research is focused on determining the location of specific translocation breakpoints and the molecular outcomes, little is known about how a translocation affects chromatin higher order 3-D structure and function. To investigate whether chromosome rearrangements or oncogene disruption have an effect on 3-D chromatin positioning, folding, or gene expression, we have employed a mouse model of progenitor B (pro-B) cell lymphoma. This model has recurrent translocations between *Myc* on chromosome 15 and *Igh* on chromosome 12 and amplification of *Myc* on another chromosome. Using fluorescent in situ hybridization, we probed a 7 Mb region distal to *Myc* that undergoes translocation to chromosome 12 in normal and tumor cells, and measured 3-D radial positioning and higher order folding. We found that the probed region of both un-rearranged and translocated chromosomes was more frequently located at the nuclear periphery in tumor cells than its counterparts in normal pro-B cells. We also found that the probed region exhibited more condensed and highly folded structures in tumor cells than normal cells. Our results also indicate that within tumor cells, the translocated region was more frequently at the nuclear periphery than the un-rearranged, even though we saw little difference in the 3-D folding between the two chromosomes. Taken together these data suggest that specific chromosome regions are repositioned within the nuclear volume in tumor cells, and can potentially influence gene expression for entire gene regions. Consistent with this, analysis of gene expression data obtained for these tumors and pro-B cells, indicate that the 7 Mb distal to *Myc* consists mainly of down-regulated genes. Further, comparison with tumors of the same genetic background but harboring amplification of the related *Mycn* oncogene, reveals a very similar pattern of expression. These data suggest that the effects of *Myc*-family oncogene activation predominate over the chromatin structural effects of translocations with respect to regional gene deregulation.

1534

Chromosomal Instability Generates Aneuploidy through a P53-Dependent Mechanism.

S. Thompson[1,2], D. Compton[1,2]; [1]Biochemistry, Dartmouth Medical School, Hanover, NH, [2]Norris Cotton Cancer Center, Lebanon, NH

Most solid tumors are highly aneuploid and many mis-segregate chromosomes at high frequencies in a phenomenon called chromosomal instability. In principal, the consequence of chromosomal instability is aneuploidy, but we recently showed that chromosome mis-segregation in otherwise diploid cells suppresses cell propagation thereby maintaining the diploid karyotype of a population. Here we explore the mechanisms that limit the growth of cells following chromosome mis-segregation. We show that aneuploid cells derived from both HCT116 (stable, near-diploid colon cancer cell line) and RPE1 (immortalized but not transformed human diploid cell line) cells propagate efficiently if grown in the presence of the p38 kinase inhibitor SB203580. Since p38 kinase-dependent stress pathways activate p53, we tested if elevated chromosome mis-segregation rates could induce high levels of aneuploidy in p53 null cells. High levels of cells with aneuploid chromosome numbers are maintained for several generations in HCT116 lacking functional p53. In clonal populations, the lack of functional p53 results in high levels of cells with chromosome numbers that differ from the mode when chromosome mis-segregation was elevated for several consecutive generations. Finally, nuclei stain positive for the cyclin-kinase inhibitor p21 in HCT116 cells following chromosome mis-segregation indicating that aneuploidy activates a delay or arrest in G1 of the cell cycle. In conclusion, we show that p53-dependent mechanisms limit propagation of aneuploid human cells, most likely through a stress activated response. Moreovoer, aneuploidy can be generated by chromosomal instability in otherwise diploid cells provided the stress response is overridden.

1535

Cell-Cell Adhesion Force Influences Tumorigenesis by Inducing Cell-In-Cell Structures.

M. Overholtzer[1], G. Normand[2], S. J. Schnitt[3], E. S. Cibas[4], R. King[1], J. Brugge[1]; [1]Department of Cell Biology, Harvard Medical School, Boston, MA, [2]HTS Core Facility, Memorial Sloan-Kettering Cancer Center, New York, NY, [3]Department of Pathology, Beth Israel Deaconess Medical Center and Harvard Medical School, Boston, MA, [4]Department of Pathology, Brigham and Women's Hospital and Harvard Medical School, Boston, MA

An important modulator of cell behavior is the balance between cell-matrix and cell-cell adhesion. For example, altered extracellular matrices can induce cell scattering and cell-cell junction disruption by increasing force resulting from integrin engagement. Conversely, we have found that under conditions of limited matrix availability, a more dominant cell-cell adhesion force mediated by adherens junctions and actomyosin can cause cells to push or invade into each other, sometimes completely, resulting in the formation of cell-in-cell structures that are a cytological feature of some human cancers. This process, termed entosis, can potentially suppress tumor growth as internalized cells are targeted for cell death by a non-apoptotic mechanism. Paradoxically, we find that entosis also provokes the development of aneuploidy, as internalized cells disrupt cell division of outer cells by blocking cytokinesis, leading to the development of tetraploids. Thus, cell-cell adhesion is a powerful modulator of cell behavior that, in addition to limiting cell scattering and tumor cell invasion, can potentially suppress or promote tumor growth by inducing the formation of cell-in-cell structures by entosis.

1536

Extracellular Matrix Signaling Regulates DNA Double-Strand Break Repair and DNA Damage Response in Breast Epithelial Cells.

A. Rizki[1], H. Helgadottir[2], B. Rydberg[3], H. Zhang[3], M. Jasin[4], M. Bissell[3]; [1]Radiation Oncology, Virginia Commonwealth University, Richmond, VA, [2]Burnham Institute, La Jolla, CA, [3]Lawrence Berkeley National Laboratory, Berkeley, CA, [4]Memorial Sloan Kettering Cancer Center, New York, NY

Extracellular microenvironment signals to maintain normalcy within a tissue. Regulation of cellular processes such as proliferation, differentiation, motility, and apoptosis contribute to tissue maintenance and have been shown to occur partly via signaling from the tissue-specific extracellular matrix (ECM). Here we show that ECM signaling also regulates some DNA damage response processes that are important for maintaining normalcy within the cellular genome. Using non-tumorigenic human breast epithelial cell lines as well as primary

mouse mammary epithelial cells we found that β1 integrin-mediated signaling regulates homologous recombinational repair of DNA double-strand breaks. Interestingly, cells with proper cell-cell junctions upregulated this error-free repair pathway, whereas single cells lacking proper tissue structure downregulated it. This is consistent with a role for ECM signaling in maintaining genome stability in a tissue-context dependent manner. Using a direct-repeat recombination substrate as well as ionizing radiation to induce double-strand breaks, we found that the histone variant γ-H2AX, the MRE11/RAD50/NBS1 complex, and RAD51 which is the recA homolog required for homologous recombination are regulated by ECM signaling. Using pulse-field gel electrophoresis, we showed that formation of breaks as well as the overall repair kinetics are not altered by ECM in either the human cell line or the mouse primary cells, supporting the observation that it is the error-free pathway choice that is regulated and not overall DNA damage induction or repair. We found regulation by ECM to be independent of cell cycle effects that have been previously reported to alter repair pathway choices. Overall, our results support a new role for ECM signaling in maintaining genome stability in a context-dependent manner, consistent with tissue-specific regulation of mutagenesis.

Minisymposium 23: Intermediate Filaments and Nuclear Lamins (1537 – 1542)

1537

Lamin Supramolecular Assembly in Health and Disease.

Y. Gruenbaum[1], N. Wiesel[1], A. Mattout[1], K. Ben-Harush[2], H. Herrmann[3], U. Aebi[4], O. Medalia[2]; [1]Genetics, The Hebrew University of Jerusalem, Jerusalem, Israel, [2]Department of Life Sciences, The Ben-Gurion University, Beer-Sheva, Israel, [3]Division of cell biology, DKFZ, Heidelberg, Germany, [4]M. E. Mueller Institute, Biozentrum, Basel, Switzerland

Lamins are nuclear-specific intermediate filament proteins (IFs) that, together with a complex set of inner nuclear membrane proteins, form a filamentous meshwork between the inner nuclear membrane and the peripheral chromatin. Lamins are involved in most nuclear activities; and mutations in human lamins or their associated proteins cause laminopathic diseases ranging from muscular dystrophies to accelerated ageing. Previous studies showed that lamin dimers form filamentous structures through 'head-to-tail' interactions, which then form paracrystalline fibers in vitro. C. elegans has a single evolutionarily conserved lamin protein, termed Ce-lamin. This lamin is unique in its ability to form under specific conditions 10-nm filaments that are distinct from cytoplasmic IFs. The 10-nm lamin filaments are composed of 3 and 4 tetrameric protofilaments, each of which contains two half-staggered anti-parallel head-to-tail polymers. The beaded appearance of the lamin filaments stems from paired globular tail domains, which are regularly spaced, alternating between 21 and 27 nm. We have also used both negative staining and cryo-electron tomography to determine how mutations in conserved residues that lead to laminopathic diseases in human, affect lamin filaments assembly. We have further analyzed the effects of these mutations on lamin organization and dynamics in vivo, by creating mutated gfp::lmn-1 transgenic strains and by performing FRAP experiments and analyzing phenotypes. Our results show that laminopathic mutations interfere with different steps of lamin filament assembly in vitro and lamin organization and function in vivo.

1538

Connections between Drosophila Lamin C and Hormone Signaling.

G. Dialynas[1], S. R. Schulze[2], S. D. Speese[3], B. Curio-Penny[1], V. Budnik[3], P. K. Geyer[1], L. L. Wallrath[1]; [1]Biochemistry, University of Iowa, Iowa City, IA, [2]Biology, Western Washington, Bellingham, WA, [3]Neurobiology, University of Massachusetts, Worcester, MA

The inner side of the nuclear envelope is lined with a network of intermediate filaments called lamins. Lamins provide structural support for the nucleus, organize the genome by making contacts with chromatin and transmit signals from the cytoplasm. Lamins are classified as A- or B-types depending on their structure and expression profile. In humans, mutations in A-type lamins give rise to a collection of diseases that exhibit tissue-specific defects, such as Emery-Dreifuss muscular dystrophy. We are using Drosophila as a model to identify tissue-specific functions of A-type lamins in development, with implications for understanding human disease mechanisms. Transgenic flies expressing wild type and mutant versions of Lamin C that are analogous to human disease forms were generated. Effects of expressing mutant versions of Lamin C in specific tissues were analyzed. Expression of wild type Lamin C does not alter nuclear architecture or viability. Amino acid substitutions within the Lamin C rod domain cause nuclear Lamin C aggregation, yet support viability. In contrast, deletion of the head domain causes no apparent nuclear defects, but results in semi-lethality when expressed specifically in larval muscle. Adult "escapers" possess malformed legs that are remarkably similar to those produced in flies defective for ecdysone signaling. Consistent with this observation, the ecdysone-induced E74 gene shows altered expression. The ability of Lamin C to directly regulate gene expression was tested by tethering Lamin C upstream of a reporter gene. Association of Lamin C with the reporter gene caused localization to the nuclear periphery and transcriptional repression. These studies demonstrate a developmental role for Lamin C in muscle, with implications for regulating gene expression through hormone signaling.

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The A- and B-Type Nuclear Lamin Networks: Microdomains Involved in Chromatin Organization and Transcription.

K. Pfleghaar[1], S. Kojima[1], C. Pack[3], R. Goldman[1], S. Adam[1], M. Kinjo[3], D. Shumaker[1], A. Goldman[1], T. Cremer[2], I. Solovei[2], T. Shimi[1]; [1]Department of Cell & Molecular Biology, Northwestern University, Chicago, IL, [2]Planegg-Martinsried, Universitaetsklinikum Essen, Essen, and Munich Center for Integrated Protein Science, Ludwig Maximilians University, Munich, Germany, [3]Hokkaido University, Sapporo, Japan

Evidence is presented that the A- and B-type nuclear lamins (LA/C, LB1/2) form separate, but interwoven fibrous meshworks within the nuclear lamina. In the nucleoplasm both types of lamins are also present in separate, more dispersed structures. Fluorescence correlation spectroscopy (FCS) reveals that nucleoplasmic A- and B-type lamins have different dynamic properties: LA/C is immobile in the lamina, but in the nucleoplasm it moves rapidly in a complex with a predicted mass of 1.3 MDa, whereas LB1/2 are immobile, both in the lamina and nucleoplasm. Silencing LB1/2 increases the mobility of nucleoplasmic LA by ~2 fold, suggesting that the B-type lamins may transiently tether LA. Silencing LB1 but not LA/C or LB2 also alters the organization of LA/C. The loss of LB1 frequently induces an increase in the

mesh size of the LA/C and LB2 meshworks. Furthermore, ~30 % of the nuclei in LB1 silenced cells also form nuclear blebs enriched in LA/C and devoid of LB2. These LA-rich blebs contain mainly euchromatic early replicating gene-rich chromosomes as revealed by double replication labeling, chromosome painting and immunostaining for H3K9me2 and AcH3. Surprisingly, BrU incorporation reveals that there is little RNA Pol II transcription within the blebs. Chromatin within the blebs is enriched in H3K4me3 and Pol IIo, but H3K36me3 is absent, suggesting that Pol II transcription in the blebs is stalled. We propose that A- and B-type lamins in the lamina and nucleoplasm form microdomains with different functions in chromatin organization and transcription. Our results provide new insights into the mechanisms underlying the changes in nuclear structure and function found in the numerous laminopathies caused by mutations in the LMNA gene, many of which are characterized by the presence of blebbed nuclei. Supported by NCI, NIA, Ellison Medical Foundation and the Deutsche Forschungsgemeinschaft.

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The IFAP BP230 (BPAG1e) Is Required for Beta4 Integrin-Mediated Regulation of Keratinocyte Motility.

K. J. Hamill, S. B. Hopkinson, P. J. DeBiase, J. C. Jones; Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL

α6β4 integrin is known to stabilize cell adhesion to matrix via its ability to nucleate the assembly of hemidesmosomes. However, α6β4 integrin also plays a role in keratinocyte migration. Specifically, β4 integrin-deficient keratinocytes display aberrant motility patterns; these cells move in a circular manner defined by arrays of laminin-332 (laminin-5) in their matrix, as opposed to the back and forward motion of wild type keratinocytes on linear trails of laminin-332. We previously demonstrated that β4 integrin regulates keratinocyte motility behavior via signaling through Rac1 to the actin-severing protein cofilin. Here, we tested the hypothesis that cytoskeletal linkers act as scaffolds for β4 integrin-mediated activation of Rac1 and cofilin. β4 integrin is known to bind a number of linker molecules, including the intermediate filament associated protein (IFAP) BP230 (BPAG1e). To study the potential role for BP230 in regulating keratinocyte migration, we generated a stable keratinocyte line exhibiting knockdown in BP230 expression levels using lentiviral shRNA technology. Knockdown of BP230 does not affect expression levels of other hemidesmosomal proteins, nor the levels of β4 integrin expressed at the cell surface. However, keratinocytes deficient in BP230 move in a circular path over arrays of laminin-332 in a manner identical to β4 integrin-deficient cells. Rac1 and cofilin activity are significantly lower in BP230 deficient cells compared to wild type keratinocytes. Furthermore, immunoprecipitation experiments demonstrate that the amount of Rac1 associating with β4 integrin is reduced in the BP230 deficient keratinocytes. Finally, the motility defect of BP230 deficient cells is rescued by inducing expression of a constitutively active form of Rac1. These data indicate that the IFAP BP230 is required for efficient regulation of keratinocyte migration by regulating the association of β4 integrin with Rac1 and thereby localizing active Rac1 to the cell surface.

1541

In Vitro and In Vivo Characterization of Human Desmin Disease Mutations.

H. Bär[1,2], S. Sharma[1,2], N. Mücke[3], H. A. Katus[1], U. Aebi[4], H. Herrmann[2]; [1]Department of Cardiology, University of Heidelberg, Heidelberg, Germany, [2]Functional Architecture of the Cell, German Cancer Research Center (DKFZ) B065, Heidelberg, Germany, [3]Division of Biophysics of Macromolecules, German Cancer Research Center (DKFZ), Heidelberg, Germany, [4]Maurice E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Basel, Switzerland

The extra-sarcomeric cytoskeleton of mammalian muscle represents a complex organized structural system that gives mechanical support for the sarcomeric working units. In addition, it connects to nuclei and organelles such as mitochondria and thereby integrates them into the functional myocyte architecture. Up to now, more than 40 mutations in the human muscle-specific intermediate filament (IF) protein desmin have been identified that cause myofibrillar disease, also referred to as desminopathy. Mutations in the alpha-helical central “rod” domain are predominant, although a number of missense mutations in the non-alpha-helical “tail” domain leading to disease have been described. More recently, mutations have also been identified in the non-alpha-helical “head” domain, which is evolutionarily less conserved than the other two domains and which is furthermore deviating significantly in the amino acid sequence from its expression and co-assembly partner vimentin. We have now investigated the biophysical behavior of five desmin disease mutations located in the “head” domain. These mutations include Ser2 to Iso, Ser13 to Phe, Arg16 to Cys as well as Ser46 to Phe and Tyr, respectively. The recombinant mutant proteins were analyzed by analytical ultracentrifugation and assembly experiments. Under low salt conditions they all sediment in the range of 4.9 to 7.0 S (wild-type: 5.5 S) indicating their association into proper tetrameric complexes. Though all of the mutants form bona fide IFs, the filaments of the Ser46Tyr mutant exhibited a slightly lower diameter than those of the other mutants and wild-type desmin (10.0 versus 12.0 nm), and the Ser13Phe as well as the Arg16Cys mutant proteins had a slight tendency to aggregate in vitro. Nevertheless, upon transfection into cultured cells, all mutants give rise to extended networks without major signs of aggregation. Hence, all five “head” domain mutants most probably interfere with proper function on a higher organizational level, i.e. with cell type-specific interactions of the desmin IF system with organizing factors of the myocyte cytoskeleton thereby eventually initiating the pathogenic process.

1542

Targeted Deletion of the Entire Keratin Gene Family in Mice Affects Global Translation and Causes Early Embryo Lethality.

T. Magin, P. Vijayaraj, C. Kroeger, U. Reuter; Biochem. and Molec. Biol., Univ. Bonn, Bonn, Germany

Onset of keratin intermediate filament protein expression marks the development of epithelia. Keratins are encoded by >50 differentially expressed genes arranged into two gene clusters. Owing to their their insolubility, keratins are essential to protect epithelia against mechanical stress. Moreover, they control cytoarchitecture, cell growth, survival and organelle transport. Understanding their function during epithelial differentiation and homeostasis has been hampered by redundancy, resulting from overlapping expression patterns and by dominant-negative effects following single gene knockouts. Here, we report on mice lacking the entire keratin multiprotein family, using a targeted gene deletion strategy. Unexpectedly, null embryos developed until ~E8.5 displaying intact epithelia which maintained intact desmosomes, reduced in numbers. Despite the absence of cytolysis, they died one day later with strong defects in yolk sac hematopoiesis and

vasculogenesis. We show that in the absence of keratins, overall protein synthesis in embryos is reduced by ~30, causing a severe impairment of growth. We will present data on the mechanisms by which keratins control protein synthesis and cell growth.

Minisymposium 24: Microtubule-based Motors (1543 – 1548)

1543

Function of Hklp2 and its Binding Partner Ki67 during Spindle Assembly.

D. Vanneste[1], M. Takagi[2], N. Imamoto[2], I. Vernos[1,3]; [1]Microtubule Function and Cell Division, CRG, Centre de Regulacio Genomica, Barcelona, Spain, [2]Advanced Science Institute, RIKEN, Hirosawa 2-1, Wako, Saitama 351-0198, Japan, [3]ICREA, Barcelona, Spain

The mitotic spindle is a highly dynamic molecular machine whose assembly and function rely on the collective activity of different classes of molecular motors. We have previously found that the kinesin-like protein Xklp2 is a plus-end directed motor with a role in centrosome separation in the Xenopus egg extract system (Boleti et al, 1996). To get further insight into the function of this KLP in mitosis we decided to study its homolog in human cells. Hklp2 is a member of the kinesin-15 family and shows a high level of identity with Xklp2 throughout its entire amino acid sequence (53%). Immunofluorescence studies in HeLa and RPE cells showed that Hklp2 is only detectable during mitosis although its expression is not cell cycle regulated. At metaphase, Hklp2 localizes along spindle microtubules and in the proximity of the aligned chromosomes. Although Hklp2 silencing has no major consequences on cell division, it promotes a higher efficiency of spindle collapse upon partial inhibition of Eg5 by monastrol. Interestingly, previous work showed that Hklp2 interacts with Ki-67, a widely used cell proliferation marker that localizes around mitotic chromosomes but whose function is still unclear (Sueishi et al, 2000). Silencing the expression of Ki67 in HeLa cells results in mild chromosome alignment defects although kinetochore function appears to be intact and both chromokinesins kid and Kif4 are correctly targeted to the mitotic chromatin. By contrast, Hklp2 localization is altered in metaphase cells lacking Ki-67. Consistently, Hklp2 localization is also altered in Ki-67 silenced cells treated with monastrol and these cells are more resistant to the collapse of the spindle poles than control cells. In addition, monastrol treated cells having collapsed spindle poles re-establish faster a bipolar spindle after monastrol washout than controls. We propose that Hklp2 participates in the establishment or maintenance of spindle bipolarity. Our data also suggest that Ki-67 participates in the establishment of a functional spindle that can efficiently align chromosomes in metaphase, a function that may at least in part be related its role in mediating the localization of Hklp2. D.Vanneste and M.Takagi, equal contribution

1544

Regulation of Cytoplasmic Dynein at Kinetochores by Phosphorylation.

J. Bader[1], J. Whyte[1], M. T. Raycroft[1], S. Tauhata[1], J. Hornick[1], G. Chan[2], E. Hinchcliffe[1], P. Vaughan[1], K. Vaughan[1]; [1]Biology, University of Notre Dame, Notre Dame, IN, [2]Oncology, University of Alberta, Edmonton, AB, Canada

Cytoplasmic dynein is thought to play important roles at spindle poles, the cell cortex and kinetochores during mitosis, however the mechanisms of targeting to each site remain unclear. Using mass spectrometry, we identified a novel mitotic phosphorylation site in the dynein intermediate chains (ICs) that controls kinetochore dynein localization. IC phosphorylation directs binding of dynein to the mitotic checkpoint protein *zw10* rather than dynactin, and this interaction is needed for dynein recruitment to kinetochores. Phosphorylated dynein associates with kinetochores from nuclear envelope breakdown to metaphase, but undergoes dephosphorylation in response to bioriented microtubule (MT) attachment and alignment at the metaphase plate. Dynein dephosphorylation shifts binding to dynactin, inducing movement of dynein and dynactin along kinetochore fibers towards spindle poles in a process known as streaming. Dynein dephosphorylation was inhibited by microtubule (MT) depolymerization, reduction of kinetochore stretch and a phosphatase-dead PP1γ mutant leading to retention of phospho-dynein at kinetochores and a reduction in poleward streaming. These defects in dynein dephosphorylation and streaming induced metaphase arrest. Although depletion of kinetochore dynactin by moderate p50(dynamitin)-expression did not affect initial recruitment of phospho-dynein to kinetochores, dynein dephosphorylation during alignment or poleward movement of dynein itself at metaphase, it did reduce poleward streaming of kinetochore checkpoint proteins at metaphase. Together these results suggest a new model for localization of kinetochore dynein and the contribution of kinetochore dynactin.

1545

"Cortical" Dynein Can Capture and Pull on Dynamic Microtubule Ends In Vitro.

L. Laan[1], J. Husson[1], M. van Duijn[1], R. Vale[2,3], S. L. Reck-Peterson[4], M. Dogterom[1]; [1]bio-assembly and organization, FOM Institute AMOLF, Amsterdam, Netherlands, [2]Howard Hughes Medical Institute, UCSF, San Francisco, CA, [3]Department of Cellular and Molecular Pharmacology, UCSF, San Francisco, CA, [4]Department of Cell Biology, Harvard Medical School, Boston, MA

The interplay between dynamic microtubules (MTs) and cytoplasmic dynein plays an important role in many different cellular processes. Dynein at the cortex positions the mitotic spindle through interaction with astral MTs. Within the spindle MTs interact with dynein at the kinetochore contributing to poleward movement of the chromosomes. Despite the apparent importance of MT-dynein interactions in these and other cellular processes, very little is known about how dynein mechanically interacts with dynamic MT ends. We have set-up different in vitro assays that mimic the MT-cortex/kinetochore interaction. Dynamic MTs grow against a micro fabricated barrier, coated with purified dynein. An optical tweezers based technique, previously developed in our lab, allows us to monitor MT dynamics and force generation while MT grow and shrink against the dynein-coated barrier. We show that dynein, artificially anchored to a barrier, dramatically alters MT dynamics. It captures MT ends, inhibits growth and induces catastrophes. Subsequent shrinking of the MT is slow compared to the free walking velocity of the motor and the free shrinking velocity of the MT. In addition, the MT-dynein connection can generate pulling forces up to 5 ± 1 pN. Our results show that the MT-dynein connection, if it is anchored to a barrier, forms a simple force-generating unit that does not require other factors to behave in a way that is similar to what is observed in living cells.

1546

Complementary Roles of LIS1 and Nude in Dynein Motor Regulation Revealed by Biochemical and Optical Trap Experiments.

M. Vershinin[1], R. J. McKenney[2], S. Gross[1], R. Vallee[2]; [1]Department of Developmental and Cell Biology, UC Irvine, Irvine, CA, [2]Department of Pathology and Cell Biology, Columbia University, New York, NY

Cytoplasmic dynein is involved in a wide range of cell movements. Recent work has identified two novel types of dynein regulator involved in some, but not all, dynein functions. LIS1 was identified as the causative gene for classical lissencephaly, a severe brain developmental disease resulting from defects in neural progenitor cell migration and division. LIS1 has been implicated in subcellular motility under high-resistance conditions, including transport of nuclei and the entire microtubule cytoskeleton (Tsai et al., 2007). NudE and its isoform NudEL are LIS1- and dynein-interacting proteins also implicated in brain development, as well as in mitotic, migratory, and transport functions. How these proteins regulate dynein function is unknown. To address this issue we used biochemical and optical trap studies (Vershinin et al., 2007) to examine the ability of these proteins to interact and the consequences for dynein behavior. We find that LIS1 alone weakly interacts with dynein, but enhances its microtubule binding affinity. In contrast, NudE binds tightly to dynein and interferes with its microtubule binding and ATPase acvitity. All together, NudE serves to recruit LIS1 to form a ternary complex with dynein with intermediate biochemical properties. Optical trap assays revealed that beads moved by single dynein/NudE complexes exhibit a dramatically reduced frequency and duration of interactions with microtubules compared with beads moved by single dynein motors alone. In marked contrast, dynein/LIS1 beads exhibited dramatic stalls under load compared with beads moved by single dynein motors alone. This effect was reversed by removal of soluble LIS1, but simultaneous or prior addition of NudE preserved stalling activity. These results identify NudE alone as an antimotility factor. LIS1, in contrast, markedly extends the duration of force producing events. NudE serves to recruit LIS1 stably to dynein. The resulting ternary complex is a substantially more effective motor under load suitable for high resistance tasks. Supp. by grants GM47434, HD40182, and GM070676.

1547

Mechanical Cross-Talk between Kinesin-1 and Cytoplasmic Dynein In Vivo.

S. Ally, A. G. Larson, K. Barlan, S. Rice, V. Gelfand; Cell and Molecular Biology, Northwestern University, Chicago, IL

Intracellular transport is typically bidirectional, consisting of a series of back and forth movements, along microtubules. During bidirectional organelle transport, kinesin-1 and cytoplasmic dynein require each other for motility, a property referred to here as cross-talk. Thus, either kinesin-1 or dynein depletion results in abrogation of bidirectional motility. We hypothesized that motor mechanics, rather than specific biochemical interactions, mediate cross-talk between these motors. To test this model, we replaced either kinesin-1 or dynein with unrelated motors that are specifically targeted to peroxisomes. In Drosophila S2 cells, we replaced endogenous kinesin-1 with an equally processive and fast motor (OSM-3, a kinesin-2 family member). In this case, OSM-3 rescues bidirectional motility of peroxisomes. This suggests specific biochemical interactions between kinesin-1 and dynein may not be responsible for cross-talk. Furthermore, speed and processivity may be essential motor properties during bidirectional transport. To test this possibility, we replaced kinesin-1 or dynein with weakly processive and slow motors. Replacement of dynein with human Ncd (a minus-end directed motor) results in accumulation of peroxisomes at microtubule plus-ends. This suggests Ncd rescues kinesin-1 driven transport towards plus-ends but cannot effectively transport peroxisomes towards minus-ends. On the other hand, replacement of kinesin-1 with Xenopus Eg5 (a plus-end directed motor) results in clustering of peroxisomes at the cell body. Here, Eg5 clearly rescues only dynein-driven transport towards minus-ends. Perhaps, neither Ncd nor Eg5 can compete with the robust motility characteristic of kinesin-1 and dynein, and thus only rescue the motor of the opposite polarity. Altogether these results show that motor mechanics, rather than biochemical interactions, mediate cross-talk between kinesin-1 and dynein during bidirectional peroxisome transport. (Supported by NIH GM-52111 to V.G)

1548

Road Signs for Kinesin Motor Transport.

K. Verhey[1,4], D. Cai[1,4], D. McEwen[2], J. Martens[2], E. Meyhofer[3,4]; [1]Department of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, MI, [2]Department of Pharmacology, University of Michigan Medical School, Ann Arbor, MI, [3]Department of Mechanical Engineering, University of Michigan, Ann Arbor, MI, [4]Biophysics Research Division, University of Michigan, Ann Arbor, MI

Despite intense investigation into how single kinesin and dynein motors convert chemical energy into mechanical force in vitro, little is known about how motors function in cells to bind to the appropriate cargo and deliver it to the proper cellular destination. To gain an understanding of how kinesin motors function in vivo, we utilized a three-tandem monomeric Citrine (3xmCit) tag and TIRF microscopy to study kinesin motors at the single molecule level in live cells. We show that single Kinesin-1 motors move preferentially on a subset of microtubules in COS cells. Preferential motility does not occur on dynamic microtubules marked by the end binding protein 3 (EB3), which decorates the plus tips of growing microtubules. Rather, retrospective immunofluorescence staining demonstrates that single Kinesin-1 motors utilize stable microtubules marked by specific post-translational modifications. Preferential motility on stable microtubules is not a general property shared by all kinesin motors as neither the kinesin-2 family member KIF17 nor the kinesin-3 family member KIF1A moved on a subset of microtubules in COS cells. Selective transport enables Kinesin-1 to carry vesicles containing the marker protein vesicular stomatitis virus (VSV)-G along stable microtubules whereas KIF17 transport of vesicles containing the voltage-activated potassium channel Kv1.5 occurs along all microtubule tracks. These results support the hypothesis that a tubulin code of post-translational modifications can direct kinesin transport events in cells.

Keith R. Porter Lecture (1549)

1549

Through the Looking Glass.

J. Gall; Embryology, Carnegie Institution, Baltimore, MD

In the Porter Lecture I will discuss studies on chromosomes and nuclear organelles carried out in my laboratory from the earliest days of the ASCB up to the present. I will emphasize the importance of specialized types of microscopes and other techniques, as well as the value of choosing the right organism or tissue for studying a specific problem.

Pre-College and College Science Education (1550 – 1578)

1550/B1

The Young Scientist Program at Washington University in St. Louis.

A. L. Buchwalter[1], M. R. Beck[4], B. L. Moss[3], J. Mosher[5], T. A. Woolsey[2]; [1]Cell Biology and Physiology, Washington University in St Louis, St Louis, MO, [2]Neurological Surgery, Washington University in St. Louis, St Louis, MO, [3]Mallinckrodt Institute of Radiology, Washington University in St Louis, St Louis, MO, [4]Biochemistry and Biophysics, University of North Carolina- Chapel Hill, Chapel Hill, NC, [5]Young Scientist Program, Washington University in St Louis, St Louis, MO

The Young Scientist Program (YSP) is a science education outreach program run by graduate and medical students at Washington University in St. Louis, Missouri. YSP aims to improve science education in local middle and high schools and attract young students to scientific careers. Student volunteers participate for reasons including a desire to participate in community outreach, to gain teaching experience, and to increase the participation of underrepresented groups in science. YSP volunteers have developed several programs to supplement the educational experience of students and their teachers. *Teaching Teams*: YSP volunteers develop and lead inquiry-driven science modules in local middle and high school science classes and after-school programs. Teaching Teams are active in the areas of Anatomy, Chemistry, Genetics & Genomics, Ecology & Evolution, Forensics, Microbiology, Neuroscience, and Physics. These science modules are modified in collaboration with teachers to integrate classroom curricula and state standards. Teaching teams have interacted directly with more than 5000 students since 1993. *Summer Focus*: Local high school students are encouraged to apply to YSP's Summer Focus program, which places the student in a summer laboratory internship at the university. Each Summer Focus Scholar is mentored by a volunteer graduate student or postdoctoral fellow, completes an independent research project, and writes a formal research paper during the eight-week internship. More than 165 local students have participated in Summer Focus. *Teacher and Researcher Partnership*: YSP initiated this program in 2001 to provide summer research and curriculum enrichment experiences for public school teachers. Teacher participants work with mentors on an individual research project and on development of science activities for their own classrooms. *Benefits and Outcomes*: YSP asks its participants to evaluate their experience in order to continuously assess the effectiveness of YSP's programs. Volunteers report gaining a variety of skills that are not formally taught during graduate and post-graduate training. Student and teacher participants report an increased interest in and understanding of scientific concepts.

1551/B2

A Saturday Academy to Prepare High School Juniors for Molecular Biology Research.

S. Walsh; Chemistry and Biochemistry, University of California, Los Angeles, Los Angeles, CA

As the diversity of our classrooms increases, the need to introduce science as a viable career option to underrepresented minority groups grows more imperative. In a program funded by HHMI, high school juniors from African-American, Hispanic, and Latino backgrounds are introduced to molecular biology research within the UCLA School of Dentistry. The curriculum consists of three parts: Saturday Academy sessions throughout the school year, a six-week summer laboratory internship, and a year of poster preparation and enrichment activities. The Saturday Academy has the following goals: to prepare students for laboratory research; to cover in-depth topics relevant to the ongoing research at the UCLA School of Dentistry; and to enhance scientific writing and communication skills. These goals are accomplished through four components: an in-class laboratory session in which students practice molecular biology techniques; a lecture by a research scientist at the School of Dentistry; a background reading assignment to introduce relevant terminology; and a take-home, kitchen-style laboratory where students can work with their families to demonstrate a particular scientific concept. Throughout the course, emphasis is placed on working together, developing and testing hypotheses, and analyzing results. In-class laboratory sessions give the students the hands-on skills and confidence to enter a research environment. Take-home laboratories expose basic scientific concepts to the broader community. Through this Saturday Academy, students sense the collaborative nature of science, begin to analyze and question data, interact with dental scientists, and become enthused about biology. Clearly, as this program progresses, more underrepresented students will see scientific research as a valid career option.

1552/B3

Neuroscience Research: a Hands on Educational Tool for Aspiring Scientists.

M. Fields; Sidwell Friends School, District of Columbia, DC

Pre-College Science Education can be an important and intriguing way to engage young scientists, as they continue to develop a passion for science. Neuroscience Research is a Sidwell Friends Upper School elective in which students experience the tedium and the triumphs of basic research. Students read original scientific literature, collaborate with scientists, as well as design, perform and refine protocols. Students also write and organize lab manuals for their projects, these are then used, edited and modified by subsequent classes. The course mimics a working lab with lab meetings, journal club, seminars and experiments. Students are exposed to current lab techniques including electrophysiology, immunocytochemistry (HuC staining in order to study neuron development), regulation of gene expression, microinjections, and microarrays. Students learn to use lab equipment such as oscilloscopes and fluorescent microscopes. The class also participates in an on-going collaboration with the National Center for Microscopy and Imaging Research (NCMIR) at UCSD. Current topics explored include the genetics and developmental biology of zebrafish, the neuroethology of weakly electric fish, and an investigation into the comparative neuroanatomy in fishes. Students also review concepts important to the core of cell biology. Neuroscience Research at Sidwell Friends School has been evolving since 1996. This poster reflects the work done this semester and provides a model that can be a starting point for similar programs in other venues.

1553/B4

Implications for Hiring Cell Biology Faculty with Education Specialties: a Cross-Disciplinary Research Study of Science Faculty with Education Specialties (SFES) in the California State University System.

K. D. Tanner[1], S. D. Bush[2], N. J. Pelaez[3], J. A. Rudd[4], M. T. Stevens[5], K. S. Williams[6]; [1]Biology, San Francisco State University, San Francisco, CA, [2]Chemistry and Biochemistry, California Polytechnic State University, San Luis Obispo, San Luis Obispo, CA, [3]Biological Sciences, Purdue University, Purdue, IN, [4]Chemistry and Biochemistry, California State University, Los Angeles, Los Angeles, CA, [5]Biological Sciences, California State University, Stanislaus, Turlock, CA, [6]Biology, San Diego State University, San Diego, CA

Efforts to improve K-16+ science education in the U.S. have included the hiring of Science Faculty with Education Specialties (SFES) - scientists who take on specialized roles in science education within their departments, including biology departments (Bush, 2006). Though SFES occupy a potentially pivotal role in reform efforts, there has been little systematic investigation of these positions. We present the first cross-disciplinary research study of SFES in the U.S., conducted in the nation's largest university system, the California State University (CSU). Of 156 CSU faculty identified as likely SFES, 103 individuals participated in a 111-question online survey (66% response rate). Of those respondents who both were tenure-track science faculty and submitted complete surveys, 59 self-identified as SFES, representing biology (34%), chemistry (24%), geoscience (14%), and physics (25%). They were 46% female, 81% white, across all faculty ranks, and trained extensively as researchers in basic science. Two SFES subpopulations were identified, those specifically Hired (H-SFES; n=31, 53%) as SFES and those who Transitioned (T-SFES; n=28, 47%) from their initial faculty roles into science education. Interestingly, more T-SFES (75%; $\chi 2$ =4.4, df=1, p=0.036) reported pursuing basic science research funding compared to H-SFES (48%), whereas more H-SFES (68%; $\chi 2$ =2.7, df=1, p=0.098) applied for science education research funding compared to T-SFES (46%). Both groups applied at equal rates (68%) for funding to support K-12 teacher development. Both SFES populations reported more formal training in basic science than in science education, more time spent on service responsibilities than non-SFES science faculty, and less institutional support for their scholarly activities. Unfortunately, almost 40% of SFES were seriously considering leaving their position, with H-SFES citing that science education efforts were not valued or understood and T-SFES citing being overworked or burned out. These research findings can inform biology departments considering SFES faculty hires and also indicate a need for national leadership to articulate expectations and establish training pathways for future SFES.

1554/B5

EUKARYON: The Use of an Undergraduate Scholarship Journal to Support Inquiry-Based Pedagogy and Strengthen a Community of Undergraduate Scholars.

S. DebBurman, S. Davis, M. Fiske, P. Smith; Biology, Lake Forest College, Lake Forest, IL

Undergraduate research and inquiry-based pedagogy are becoming increasingly crucial components in colleges and university curricula in the United States. Providing undergraduates the opportunity to publish their scholarship serves as a pedagogical tool to increase scientific literacy and motivation for scientific careers. Spurred by an NSF-CCLI grant in 2004, the Lake Forest College Biology Department accordingly developed a peer-reviewed undergraduate research journal of life science scholarship entitled Eukaryon (http://www.lakeforest.edu/eukaryon). In each annual issue, Eukaryon publishes scholarship in any sub-discipline of the life sciences that students have produced within the department's research-rich undergraduate classrooms and faculty labs, in a variety of scientific and journalistic formats. Here, we detail three aspects of the journal's development: 1) how it is exclusively governed, published, and financed by undergraduates; 2) how the journal establishes and improves on its content and format; and 3) specific strategies that maintain publication selectivity and integrates the journal with student course work and faculty-student collaborative scholarship. Finally, we discuss how assessment of our journal's initial impact on a department's curriculum and its community of student scholars is shaping future directions for journal growth and curricular outcome. Given the rapid success Eukaryon has enjoyed at our institution, we encourage the adaptation and implementation of such journals at undergraduate institutions that seek to further strengthen their community of students as scholars and their inquiry-based pedagogy.

1555/B6

Nature Education: a Community Approach to Life Science Education.

K. Shaw[1], K. Zhaurova[1], M. Powers[2], V. Savkar[2]; [1]Nature Education, Nature Publishing Group, Washington, DC, [2]Nature Education, Nature Publishing Group, Boston, MA

The face of undergraduate education will change dramatically in the next 10 years as students and professors become more dependent on online, open-source technologies and content. Nature Education: Genetics is an example of how one publisher is proposing to innovate how students learn, communicate and inquire about science in an online learning environment. The learning environment provides students with a tool that allows extensive filtering of search results to personalize student learning style and objectives. By combining a rigorous search, collaborative and dynamic learning environment, and an independent editorial board, we are actively creating an active, immersive and cooperative educational environment with up-to-date content placed in a pedagogically effective context. Providing authoritative community-authored, community-reviewed content in this environment will allow professors and their students to personalize their teaching and learning.

1556/B7

Integration of Bioinformatics experience into a Capstone Cell Biology Course.

D. J. Matthes; Genetics, Cell Biology and Development, University of Minnesota, Twin Cities, Minneapolis, MN

While stand-alone courses in bioinformatics have become increasingly common at the undergraduate level, most biology students graduate without taking such a dedicated course. In an effort to increase the number of students who are aware of the importance of computational methods and have carried out bioinformatics analyses of their own, we have introduced bioinformatics-based activities in our capstone cell biology course. We hypothesized that these activities would not only provide students with first-hand experience working with bioinformatics data and introduce them to the power and breadth of bioinformatics resources, but would also enhance their learning of central concepts in cell biology. To this end we have tested whether students learn concepts in cell biology that are embedded in the bioinformatics

problems as well as or better than similar students in the class who are introduced to the same concept in the context of a different type of problem. Here we present the cell biology-focused bioinformatics activities we've developed, the methods we used to assess student understanding of each pertinent cell biology concept, and show the impact that bioinformatics-based activities has on acquisition of cell biology knowledge.

1557/B8

Investigative Cases and Student Outcomes in an Upper Division Cell and Molecular Biology Laboratory Course at a Minority Serving Institution.

J. Knight[1], R. Fulop[2], L. Márquez-Magaña[1], K. Tanner[1,2]; [1]Biology, San Francisco State University, San Francisco, CA, [2]SEPAL: Science Education Partnership and Assessment Laboratory, San Francisco State University, San Francisco, CA

Active learning strategies are increasingly being integrated into college level science courses to make material more accessible to all students and to improve learning outcomes. One active learning pedagogy, case-based learning (CBL), was developed as a way to both enhance engagement in the material and to accommodate diverse learning styles. Yet adoption of CBL approaches in undergraduate biology courses has been piecemeal, in part because of the perceived investment of time required. Furthermore, few CBL lesson plans have been developed specifically for upper division laboratory courses. Here we present four cases that we developed and implemented for a senior cell and molecular biology laboratory course at San Francisco State University, a minority serving institution. We used both written and verbal assessments to gauge learning outcomes and attitudinal responses of students over two semesters. Students responded positively to the new approach and appeared to meet the learning goals for the course. Most said they would take a course using CBL again. These case modules are readily adaptable to a variety of classroom settings.

1558/B9

The Trouble with Transfer.

W. Bradshaw[1], K. Groneman[2], J. D. Bell[3]; [1]Microbiology and Molecular Biology, Brigham Young University, Provo, UT, [2]Biology, Brigham Young University, Provo, UT, [3]Physiology and Developmental Biology, Brigham Young University, Provo, UT

When the objectives for courses in biology include the acquisition by students of skills in scientific reasoning, it is hoped that these thinking tools will carry over to be applied in novel settings: to new concepts, future courses, other disciplines, and non-academic pursuits. This is the educational concept of transfer. Our efforts over many years to design effective assessment questions that measure competence in both deep understanding of conceptual principles and the ability to draw valid conclusions from experimental data have had at least one disquieting result. The transfer performance of many otherwise capable students is not very satisfactory. On the assumption that the prompts for our transfer problems might be at fault, we experimented with multiple versions that differed in wording or the biological setting in which the concept was placed. Performance on the various versions did not change significantly. We are led to investigate two potential underlying causes for this problem. First, like any other important scholastic trait, the ability to transfer requires directed practice through multiple iterations, a feature absent from most courses. Second, conventional strategies for how to study and prepare for exams generate a perspective about learning that is inimical to transfer. Students sometimes see exams as tests of gamesmanship; "Teachers are trying to outsmart me with trick questions." Post-exam conversions can be very litigious: "But it's not clear what you wanted!" We recommend the pedagogical use of "fuzzy" problems which place on the learner the responsibility to define the appropriate scope for inquiry and improve one's ability to acquire the kind of precise and comprehensive understanding that makes transfer possible.

1559/B10

Interactive Computer Simulations for Teaching Cancer Cell Biology.

N. Dokken[2,3], C. Pennell[2], T. Otter[1], J. Habig[1]; [1]Crowley Davis Research, Eagle, ID, [2]Masonic Cancer Center, University of Minnesota, Minneapolis, MN, [3]John F. Kennedy High School, Bloomington, MN

We have created computer models and simulations for teaching fundamental cell biology, principles of experimental design, and data analysis associated with mechanisms of carcinogenesis. Multicellular virtual tissues or clusters of cells arise by growth, division, death and differentiation of one or a few starting cells. Consistent with Hanahan and Weinberg (Cell 100:57), the models are sensitive to targeted mutations that must accumulate in a normal cell to transform it into a tumor cell with unrestrained growth. For instance, sequential mutations that render a cell insensitive to anti-growth signals, self-sufficient in growth factor, non-senescent, and resistant to apoptosis produce virtual cells that grow and divide unchecked. Students can study the effects of induced mutations on any cell's gene expression and intracellular resources, monitor growth or division potential, track cell lineages (to assay the behavior of normal or mutant cells), learn about processes that affect cell proliferation in vivo by analysis of metabolic regulatory networks that control emergent cell behaviors, or explore more specialized topics such as immunosurveillance and tumor detection. In addition, we have built computer models of in vitro development of mammary acini, B and T cell lineage commitment, viral infection and replication, and pathways of apoptosis. Genes and gene expression, molecules and their locations, metabolic networks, and cellular processes (adhesion, signaling, secretion, turnover of components, growth, division, etc.) are represented in the simulations. The modeling platform is accessible from any Internet-capable workstation via an intuitive graphical interface. Because of its versatility, compatibility with readily available computing equipment and ease of use, this approach can be integrated with a variety of teaching styles and settings in secondary and college education. Sample simulations are available at http://www.cdres.com/ascb08educ.

1560/B11

Boston College Biology Commons: a Website for Integrating Advanced Laboratory Courses and a Community Resource for Educators and Students.

C. OConnor; Biology, Boston College, Chestnut Hill, MA

Like many departments with high student to faculty ratios, the BC Biology Dept. faces the challenge of providing an original research experience to large numbers of undergraduate biology majors. Therefore, the department has developed advanced laboratory classes that

involve students in functional genomics research, using clone and data resources from the Yeast Genome Project as starting materials. Four advanced laboratory classes in cell biology, molecular biology, and biochemistry develop and test hypotheses addressing the physiological significance of three putative methionine sulfoxide reductases (Msrs) in the budding yeast, Saccharomyces cerevisiae. One of the three enzymes has been biochemically characterized, a second is annotated as a putative Msr, and the third is listed as a protein of unknown function. To integrate activities between classes and between semesters, we have developed a website, (BC)2, Boston College Biology Commons. The file formats used on the site are widely used, platform-independent, and likely to readable for the forseeable future (html, pdf, swf). This enables students to use the material regardless of the type and location of the computer that they are using. The website consists of four divisions. The first division is an area in which students submit reports of their results, after peer review from other students. The second division consists of narrated tutorials, many of which instruct students on the use of online databases and bioinformatics tools. These tutorials consist of annotated screen shots with voice over. Tutorials are constructed with Macromedia Captivate and output as movies in flash .swf format. The third section is devoted to laboratory protocols. Posting this material on the Web has several practical advantages. Files are easily updated, so students can benefit from the latest refinements made to the procedures. Students have 24/7 access to the files, which is important to students working in the lab outside of normal class hours. The fourth division of the site consists of hyperlinks to sites that students will use in their research. The site is available as a resource to the scientific community.

1561/B12

Using a Cell Model Project in a Cell Biology Course.

K. Shannon[1], J. Bell[2]; [1]Biological Sciences, Missouri University of Science and Technology, Rolla, MO, [2]Natural Sciences, Fayetteville State University, Fayetteville, NC

Here we describe the use of a cell model project in an introductory Cell Biology course. Students are required to create a 3D model or website of a unicelluar or differentiated eukaryotic cell with a short written description. The goals of this assignments are for students to: Show how differentiated cells are specialized for a specific function Depict the structure of cellular organelles Describe how certain organelles contribute to the cell's function Instructional benefits to using the cell model project are to encourage creativity and hands-on learning, and to provide an opportunity for tactile/kinesthetic learners. This project is at the third level of Bloom's taxonomy, application. The model project is designed for students to take what they have learned in class about cells and organelles, and apply it to build a model of a specialized cell and be able to produce a description about how the cell's structure is important for its function. We will present our experiences using the cell model project, examples from students, and a grading rubric. We have attempted to evaluate the usefulness of the project by 1) asking students "What is one thing you have learned from the cell model project?" at the end of two semesters at Fayetteville State University, and 2) having students complete an evaluation to determine the effectiveness of the project for achieving specific course objectives at the end of one semester at Missouri University of Science and Technology. Student responses indicated that the project increased their understanding of the function of organelles, the complexity of cells, that cells have varied shapes, and how cells are specialized.

1562/B13

Restructuring Molecular Animations to Improve Learning.

J. Honts, M. Reinke; Biology, Drake University, Des Moines, IA

Molecular animations had previously been created to help students understand complex cellular phenomena, such as signaling pathways. Based on formative evaluations of these animations, the animations were re-designed in order to improve their efficacy. The G-protein signaling pathway had originally been rendered as a series of steps, presented in a continuous manner without pauses. The activation and inactivation mechanisms in these pathways were depicted, and a comparison was made of G-protein signaling pathways in two different types of cells. The original animations emphasized realism in terms of form and movement, but they were found to lack context and a clear sense of direction. Taking cues from the visual arts and cognitive science, the animations have been re-engineered to address these specific problems. In the revised animations, context and direction have now been made explicit. The new animations first show an overview of the entire pathway before focusing on the individual molecules and mechanisms in the pathways. As a result, the viewer has now been given cues as what's coming next, and in which direction to look for future changes. The context of these signaling pathways within the human body has been detailed at the beginning of the animations, and the relevance of these signaling pathways to human physiology has been described. In addition, the individual animations have been segmented into discrete blocks to take into account the way the brain breaks a continuous process into a series of steps. In segmenting the animations, the roles of specific molecules in the pathway have been highlighted, one at a time. Additional time has been allotted for the viewer to assess the information presented. Finally, a new assessment tool has been developed to test the effectiveness of the alterations that have been incorporated in these new animations. These animations will be made available at the conclusion of the current study. Future modifications are also planned, including the addition of sound effects, narration, and interactivity.

1563/B14

Teaching and Learning of the Biological Sciences.

I. Novak; [1]Facultad de Ciencias Medicas, Instituto de Biologia Celular, Universidad Nacional de Cordoba, Cordoba, Argentina, [2]Biologia Celular, Histologia y Embriologia, Facultad de Ciencias Medicas, Universidad Nacional de Cordoba, Cordoba, Argentina

In Meaningful Learning the previous knowledge composing the cognitive framework serves as an anchorage to the new construction of knowledge (Ausubel, 1976). From a constructivist perspective, (Ausubel et al 1983) both teaching and learning are conceived in a dialectic way considering the subject's inner structure. The learning as a reframing with an holistic view, the whole does not constitute a mere sum of parts (contribution of the Gelstat's theory). According to Ausubel for a meaningful learning some conditions related to the content and inner disposition of the student should be considered. Even though the meaningful knowledge can be acquired either by discovery or reception, in academic environment knowledge is generally attained by reception. Thus, it is crucial to consider the meaningful conditions: a) Affective meaning: initial predisposition for active reception, b) Cognitive Meaning: activation of previous knowledge, c) Logical Meaning: new

knowledge structuring process considering the inner logical structure of the object of study (central and interrelated concepts of such discipline). "Sequential organizers" are used in the process of content structuring. In this way, the gradual differentiation and integrating reconciliation in the cognitive structure takes place. Thus, learning implies a "framing of a clear, stable and organized body of knowledge which allows future incorporation of information in that field" (Peme de Aranega, 1987). The area of knowledge in the Biological Sciences is complex, the levels of organizations are a very complex hierarchical organization (Curtis et al, 2008) and this complexity should be considered. Each of these levels is inclusive and represent more than the sum of previous ones. The Complexity Theory (Morin, 1995) may be the appropriate one to interpret this object of study whose interactions and uncertainties are relevant and demands several view points for teaching and learning.

1564/B15

The Gene: What Is It?

K. Polacek[3,1], J. C. Walz[1,2], S. Elrod[1,2]; [1]Center for Excellence in Science and Mathematics Education, California Polytechnic State University, San Luis Obispo, CA, [2]Biological Sciences Department, California Polytechnic State University, San Luis Obispo, CA, [3]Fielding Graduate University, Santa Barbara, CA

To the learner, the language of genetics is filled with complicated terminology and jargon that experts take for granted. Research has shown that students have difficulty fully understanding the nature and function of genes. For example, students naively believe that genes encode single physical traits, and frequently lack a complete understanding of the ubiquity of genetic information and its implications for gene regulation. Given that textbooks are a common reference source, we reviewed features used to describe the term gene in 6 current texts. Our preliminary analysis revealed considerable inconsistencies across texts that may reinforce student misconceptions about genes. More than half of the glossaries included two distinct definitions: one describing the hereditary function of genes and one describing the molecular nature of genes. Indexes contained between 4 and 52 headings and sub-headings for the term gene, leading students to multiple pages throughout the book to uncover the meaning of the term; one text even devoted an entire chapter to "Definitions of the Gene". Texts contained multiple figures of genes ranging from regions within chromosomes to examples of specific DNA sequences. In addition, figure legends contained any combination of terms like gene, allele, exon, intron, promoter, and protein. These observations reflect the complex nature of the gene and our continually evolving understanding of it. In order for students to move toward expert understanding, we believe that there should be a single place where they can find an explicit description of the complex nature of the gene. Using research that documents specific student misconceptions, we will suggest ways in which textbook features might be modified to enhance both the teaching and learning of genetics.

1565/B16

The Genetics Concept Inventory (Genci) Version 3.0: Identifying and Addressing Student Misconceptions.

S. Elrod[1,2], B. D. Bartel[1], J. C. Walz[1,2], K. Polacek[3,1]; [1]Center for Excellence in Science and Mathematics Education, California Polytechnic State University, San Luis Obispo, CA, [2]Biological Sciences Department, California Polytechnic State University, San Luis Obispo, CA, [3]Fielding Graduate University, Santa Barbara, CA

College students often graduate with the same scientific misconceptions they had when entering high school. Misconceptions about genetics have been reported in the literature over the past 25 years. Instructor knowledge of misconceptions is an important step in creating effective learning experiences. We developed a genetics concept inventory (GenCI) as a diagnostic tool to help instructors identify and address student misconceptions. Initial versions of the GenCI were given to nearly 400 introductory and upper division genetics students at a large public university in pre- and post-test formats. These results revealed that college students at all levels: 1) have a superficial understanding of alleles, chromosomes, gene function, gene regulation and the ubiquity of genetic information within organisms; 2) cannot integrate their understanding of alleles with other concepts such as ploidy; and 3) have a much better grasp of transcription than translation. Results from student responses, interviews and teaching interventions also suggest that there are at least two kinds of misconceptions among students: naïve conceptions and alternative conceptions. Naïve conceptions are underdeveloped ideas that may or may not be correct (e.g., genes encode proteins). They may originate from the use of terms that also have common meanings (e.g., sister, daughter), historical terminology that may over-generalize (e.g., chromosome), assumed expert knowledge (e.g., students should already know this), or figures in textbooks (e.g., chromosomes as X-shaped structures); they should be easily corrected with appropriate instruction. Alternative conceptions are mental models that are not aligned with current scientific evidence, and are unique to particular individuals but may be commonly held (e.g., not all cells within an organism contain the same genetic information); they are often highly resistant to instruction. We will present the current, validated version of the GenCI and examples of how it can be used by instructors to identify student misconceptions in their own classrooms.

1566/B17

The Genetics Concept Assessment: a New Instrument for Gauging Student Understanding of Genetics.

M. K. Smith, W. B. Wood, J. K. Knight; Science Education Initiative, MCD Biology, Univ. of Colorado, Boulder, Boulder, CO

We have designed, developed, and validated a Genetics Concept Assessment (GCA), to test achievement of nine broad learning goals in majors and non-majors undergraduate genetics courses. Written in everyday language with minimal jargon, the GCA is intended for use as a pre-test and post-test (embedded in the final exam) to measure student learning gains. The GCA has been reviewed by genetics experts, validated by student interviews, and taken by over 600 students at three institutions. Among the 25 questions, percent correct answers on the pre-test (item difficulty index, P) ranged from 10% - 70%. Mean scores on the pre- and post-tests were, respectively, 40% and 74% for students, and 77% and 88% for graduate teaching assistants and undergraduate learning assistants in the course. The mean score for experts from 10 institutions was 93%. The 25 questions varied in ability to discriminate between students whose overall test scores identified them as "strong" or "weak" (item discrimination index, D) on both the pre- and post-tests. For standardized tests such as the SAT, such variability is considered inappropriate. However, for a pre-test/post-test concept inventory such as the GCA, which assesses understanding in several different areas, variable D values allow a distinction between concepts that are well learned or poorly learned during the course, identifying areas where instruction was successful and areas that may require improvements. For example, two questions addressing the learning goal

"be able to describe the processes that can affect the frequency of phenotypes in a population over time" consistently showed high positive D values on both the pre- and post-tests, suggesting that the mechanism by which new alleles arise in a population remains a difficult concept for all but the stronger students, even after a semester of genetics instruction.

1567/B18

The New Genetics: Electronic Tools for Educational Innovation.

S. Tobin[1], E. Johnson[2], M. Huxley[3], A. Boughton[4]; [1]Center for Biomedical Ethics, Stanford University, Palo Alto, CA, [2]Bio-Link Program, City College of San Francisco, San Francisco, CA, [3]Consultant, Bioscience Topics, Ventura, CA, [4]Twisted Ladder Media, San Francisco, CA

The intellectual merit of this project involves the goal of engaging student interest in cutting edge science by creating highly integrated educational materials that combine genetic and genomic science, technological concepts, environmental, agricultural, and biomedical applications, and societal and ethical issues. The broader impact is derived from the working hypothesis that such materials can appeal to students at several stages of undergraduate education, in a wide range of courses, and at diverse educational institutions. Thus we believe that the activities presented in this proposal will result in the creation of educational materials that are innovative and effective, and that the evaluation program will enable us to detect workable educational approaches, to target needed improvements during revisions, and to identify strengths and limitations under widely varying classroom conditions. Project Development Web Site: http://thenewgenetics.stanford.edu The project website makes additional information about the project available to the public. In addition, project faculty, students, and project personnel can use a login and password to gain access to the materials to be evaluated, the draft image bank, the evaluation instruments, and human subjects documents. Current Educational Units under Evaluation 1. On-line unit on mutation-structure-function in humans using a mutation causing cystic fibrosis 2. On-line unit on human migrations with a tutorial using on-line public bioinformatics resources to follow human migrations tracked by polymorphisms in the cerumen (ear wax) gene 4. Workbook problem set involving estimation of hereditary breast cancer risk 5. Workbook exercise involving DNA fingerprinting to identify stolen hardwood tree 6. Workbook exercise tutorial linked to courseware unit on human migrations 7. Workbook discussion and debate question on pre-implantation genetic diagnosis 8. Workbook launching pad on human genetic conditions Contributions We believe that the results of this project will be relevant to a broad range of undergraduate courses and experiences and will make genetic and genomic sciences exciting and compelling for students.

1568/B19

The Genomics Education Partnership.

S. Elgin[1], S. C. Bhalla[2], V. Chandrasekaran[3], C. Coyle-Thompson[4], Y. Gosser[5], L. L. Mays Hoopes[6], E. C. Regisford[7], W. Leung[1], C. Shaffer[1], D. Lopatto[8]; [1]Biology, Washington University, St. Louis, MO, [2]Computer Science & Engineering, Johnson C Smith University, Charlotte, NC, [3]Biology, St Mary's College of California, Moraga, CA, [4]Biology, California St Univ - Northridge, Northridge, CA, [5]Chemistry, City College of New York, New York, NY, [6]Biology, Pomona College, Claremont, CA, [7]Biology, Prairie View A&M University, Prairie View, TX, [8]Psychology, Grinnell College, Grinnell, IA

The advent of genome sequencing technology has opened up new ways to understand how genes and genomes are organized, both critical parameters in the regulation of gene expression. The massive amount of publicly available data generated by the new sequencing machines has also created new opportunities for student-scientist research partnerships. Sequence improvement and annotation, both labor-intensive processes, are now often the bottleneck for effective use of sequence data. With appropriate training, undergraduate students can provide the needed human input. The Genomics Education Partnership (GEP) brings together faculty from over 40 colleges and universities in a collaborative project to provide their students with a research experience in genomics, making research a key part of a genetics/genomics lab course. The first project undertaken by the GEP is the sequence improvement and annotation of the dot chromosomes of several Drosophila species. This unique domain has properties of both euchromatin, including normal gene density, and heterochromatin, including a high density of repetitious DNA- remnants of transposable elements and retrotransposons. Undergraduates improve the posted sequence data to an error rate of less than 1:1000 bp, and generate carefully annotated gene models; the pooled data is being used for analysis of the chromosome as a whole, including comparisons among different Drosophila species. Students appreciate the opportunity to make novel contributions to the scientific database, and that their work will result in a scientific publication. A post-course survey of student learning gains yielded results similar to those of students involved in a full-time summer research experience. We find that genomics research is a rewarding way for us to teach, and a rewarding way for our students to learn. We are looking for additional schools to join us, and additional partners from the scientific community to pose meaningful annotation problems for the students to tackle. Funded by grants from HHMI and NIH.

1569/B20

Implementation of Peer-Led Team Learning in General Biology.

T. W. Hendrickson, L. S. Blumer; Biology, Morehouse College, Atlanta, GA

Peer-Led Team Learning (PLTL) is a problem-solving method that has been traditionally used in Chemistry courses; at Morehouse College we have adapted this model for the first semester of our two-semester introductory General Biology course. In 90-minute weekly workshops, students are engaged in team efforts to solve problems. The problems are based on concepts covered in the lectures immediately preceding the workshops. These weekly workshops are led by upperclassmen, peer leaders, who have previously done well in the course. Peer leaders are trained in pedagogy, conflict resolution, and classroom management. Additionally, the coordinating instructors who oversee this initiative provide supplemental training for the peer leaders throughout the semester. The traditional teaching model centers on a lecturer disseminating information to a group of students in a classroom with limited opportunity for student involvement. While this remains a part of the teaching process, there are more effective ways of engaging the students, such as fostering active learning. PLTL offers students an opportunity to take ownership of the material, thereby allowing them to assume a more active role in the process. Since the implementation of PLTL the proportion of students successfully completing this gate-keeper course has significantly increased. Moreover, the upperclassmen who serve as peer leaders are able to further develop their leadership skills while gaining a deeper understanding of the course material. Overall, the implementation of PLTL in General Biology has been an enormous success and we are in the process of developing additional materials for implementation in the second semester introductory course and in the upper-level courses.

1570/B21

Improving Student Data Analysis Skills.

A. Wilson; Biological Sciences, Benedictine University, Lisle, IL

Students often demonstrate a lack of confidence when asked for conclusions to be drawn from a dataset. To address this need, students were provided with datasets from the scientific literature from which they could make observations and conclusions on a regular basis. After several presentations by the professor, students were asked to present datasets to the class. This presentation required students to compose questions to lead their classmates through the dataset, facilitate answering these questions with the class working in groups of two or three, and in a formal PowerPoint presentation, inform their peers about techniques used to generate the data and lead discussion about the main points of the dataset. Students were asked to use a student response system (clickers) to actively engage students in the answering of questions during the presentation. Student feedback to the activity was positive. Students gained confidence in their analysis skills and got past their overwhelming apprehension of addressing the primary literature. As student presentations had to be approved for correctness prior to class, confidence in analysis skills was reinforced during interaction with the professor. Analysis of data analysis skills at the end of the semester indicated that a majority of the students could make valid conclusions of cell biology data. Additional benefits were that students became less intimidated with analyzing and understanding primary data, students learned about techniques for studying cell biology and students were actively engaged in the course material.

1571/B22

Designing Controls for Classroom Research & Measuring How Students Learn Scientific Research Skills.

E. Stone; Cal Teach, UC Berkeley, Berkeley, CA

Controlled research on what students are learning in their coursework is generally difficult to accomplish while teaching. I designed a study for my own classroom that enabled me to easily test the implementation of two different assessment strategies at the same time using paired classes that served as controls for each other, switching groups mid-year so that all students received the same instruction by the end. I have been interested in creating assessment tools for the high school and college life sciences classrooms that both measure and promote the use of basic science skills important for scientific research. In the laboratory, a scientist must be able to formulate hypotheses, analyze and interpret data, make models, collaborate with other scientists, and perform numerous other scientific problem-solving skills. I developed a Group Collaboration Rubric and a list of "A to Z Science Skills" to make the process of scientific thinking and experimentation explicit to students. I periodically assessed students with a Group Collaboration Quiz and a Science Skills Quiz to measure their performance. Additionally, I did a pre-, midcourse- and post-assessment with a Science Skills Questionnaire to measure how student understanding of science skills and collaboration changed from the beginning to end of the year, using a Knowledge Integration framework to score responses. I found that students expressed an increasing awareness of who they are as scientists, and developed a more specific vocabulary for discussing their strengths in science and group work. Moreover, these new assessment approaches enabled me to work individually with struggling students to help them master critical skills. Not only was I able to satisfy myself that the new assessment tools I put into place were effective, but I found that this teacher research improved my own teaching practice. I am currently developing these tools further to use with undergraduates in math & science teacher education to guide them in developing research skills during a laboratory summer internship. The study design described here offers a strategy for managing effective educational research while teaching high school, undergraduate or graduate classes.

1572/B23

How Biology Students Benefit from an Interdisplinary Peer-Mentoring Cohort.

M. C. Hart[1], R. Bates[2], D. Nykanen[3], M. Rahman[4]; [1]Biological Sciences, Minnesota State University, Mankato, MN, [2]Computer Science, Minnesota State University, Mankato, MN, [3]Mechanical and Civil Engineering, Minnesota State University, Mankato, MN, [4]Mathematics, Minnesota State University, Mankato, MN

In conjunction with a National Science Foundation-sponsored scholarship program, we have implemented an interdisciplinary peer mentoring support system for students enrolled in one of the following majors: biology, mathematics, computer science, and engineering. The purpose of the program is to: 1) increase the number of underrepresented students in these disciplines, 2) increase the number of women in the programs, 3) improve student retention, 4) seek ways to attract non-traditional students, and 5) partner with community colleges to promote and provide educational opportuniteis over a wider geographic area. Key components of the program are the integration of research and academic experiences, a broadened participation of underrepresented groups and an improved infrastructure for meeting student needs. Successes of the program include the scholarship cohort, the advising program and the associated seminar. Feedback from anonymous surveys as well as semi-structured interviews of participating students indicates that key benefits are being a member of an interdisciplinary group, learning, whether major-required content or not, active participation in scholarly activity and defining professional goals.

1573/B24

Improved Performance on Clicker Questions Following Peer Discussion Results from Gains in Conceptual Understanding, Not Just from Peer Influence.

M. K. Smith[1], W. B. Wood[1], W. K. Adams[2], C. Wieman[3,2], J. K. Knight[1], N. Guild[1], T. Su[1]; [1]Science Education Initiative, MCD Biology, University of Colorado, Boulder, CO, [2]Dept. of Physics, University of Colorado, Boulder, CO, [3]Dept. of Physics, Univ. of British Columbia, Vancouver, BC, Canada

Personal response systems or "clickers" are promoted as a means to increase student learning in large undergraduate science courses. When students answer a clicker question individually, discuss it with their neighbors, and then re-vote on the same question, the percentage of correct answers typically increases. This outcome could result from gains in understanding during discussion, or simply from peer influence of knowledgeable students on their neighbors. To distinguish between these alternatives in an undergraduate genetics course, we followed the above exercise with a second, very similar (isomorphic) question on the same concept that students answered individually. Our results indicate clearly that peer discussion enhances understanding, even when none of the students in a discussion group originally knows the correct answer.

1574/B25

An Analysis of Student Questions in a Cell Biology Laboratory.

E. Keeling[1], K. M. Polacek[2], E. L. Ingram[3]; [1]Biological Sciences, California Polytechnic State University, San Luis Obispo, CA, [2]Library, Fielding Graduate University, Santa Barbara, CA, [3]Applied Biology & Biomedical Engineering, Rose-Hulman Institute of Technology, Terre Haute, IN

Asking questions is an essential component of the practice of science, but question-asking skills are often underemphasized in science education. In this study, we examined questions written by students as they prepared for laboratory exercises in a senior-level cell biology class. Our goals were to discover 1) what types of questions students asked about laboratory activities, 2) whether the types or quality of questions changed over time, and 3) whether the quality of questions or degree of improvement was related to academic performance. We found a majority of questions about laboratory outcomes or seeking additional descriptive information about organisms or processes to be studied. Few questions earned the highest possible ranking which required demonstration of extended thought, integration of information, and/or hypotheses and future experiments, although a majority of students asked such a question at least once. We found no correlation between types of student questions or improvement in questions and final grades. Only a small improvement in overall question quality was seen despite considerable practice at writing questions about science. Our results support the findings of other researchers that directed instruction may be required to improve student ability to generate higher-order questions.

1575/B26

How to Lose the Lectures without Losing the Learning: Team-based Learning in Introductory Biology.

R. Wright[1], V. Pompei[2], M. Decker[2], D. Wassenberg[2], S. Wick[2,3]; [1]Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, [2]Biology Program, University of Minnesota, Minneapolis, MN, [3]Plant Biology, University of Minnesota, St. Paul, MN

Undergraduates in the College of Biological Sciences at the University of Minnesota take a year-long introductory biology course with lab, Foundations of Biology. Instead of listening to lectures, each section of up to 117 students meets for two 2-hour "Concept Labs" (D. Udovich, Oregon) each week in a high-tech, "SCALE-UP" style classroom with round tables. Emphasis is on team work that promotes higher order skills (Bloom's levels: application, analysis, synthesis, evaluation). Important features of the course include: sustained emphasis on formative assessment, immediate feedback, and group discussion to arrive at understanding; opportunities to revise work based on peer and instructor feedback; explicit integration of university and college learning outcomes; regular use of concept maps and other tools to make explicit the emerging accuracy and complexity of their understanding. In the first semester, each unit of instruction begins with a Learning Readiness Quiz that holds each student accountable for learning the basic concepts from reading assignments (text book, on-line tutorial, scientific literature) before class. Students spend the remaining class time developing their critical thinking/problem solving skills though activities that include computer simulations, analysis of MCAT questions, data analysis and interpretation, and case studies. A large component of the students' grades is based on Team Synthesis & Evaluation Projects. For example, to help students synthesize evolutionary biology and cell structure, teams are tasked with advising a pharmaceutical company about ideal targets for developing new antibiotics for XDR-tuberculosis. This teaching model transforms the relationship instructors have with their students from one of "information deliverer" to "thinking and learning coach." As a result, most of the instructor work involves setting up projects or problems and evaluating student work. We will present strategies and tools such as team projects, study guides, grading rubrics, team evaluation strategies, intellectual property notebooks, etc., that help structure this "lectureless" course into a significant learning experience for students.

1576/B27

Nationwide Testing of Active Learning Classroom Materials for Biochemistry.

J. Loertscher, V. Minderhout; Chemistry, Seattle University, Seattle, WA

As the scope of biochemistry becomes larger and more interdisciplinary, the question of what students should learn in a biochemistry course becomes a significant challenge. Although a set of biochemical concepts are fundamental, decisions must always be made on what content to cover or omit when teaching a course. One solution to this problem is to make acquisition of content-independent skills central in biochemistry courses. Such skills, including the ability to dissect a problem, the ability to collaborate with others, and the ability to think in an integrated manner, enable students to be life long learners. Biochemistry at Seattle University has been taught without lecture using a structured small group format for the past ten years. This format enables instructors to connect learning of biochemistry content to practice of essential skills. We have seen evidence of conceptual gains and skill acquisition using a variety of assessment instruments in our own classroom. We are currently conducting an NSF-funded effort to have our active learning materials implemented and evaluated on a national level in preparation for wide dissemination. Feedback from participating beta testers from a variety of institutions including a large research university show that our activities can be used in combination with other teaching approaches such as lecture and are adaptable for use in many different classrooms.

1577/B28

Pursuing Authentic Research in an Introductory Biology Teaching Laboratory.

B. Couch, R. Wright; College of Biological Sciences, University of Minnesota, St. Paul, MN

Biology 2004 is the laboratory component of the second semester of "Foundations of Biology," a year-long lecture/laboratory course required of all undergraduate majors in the College of Biological Sciences at the University of Minnesota. Students in Biology 2004 engage in an authentic research project that is intimately connected to the research interests of a faculty member in our College. As a result, resources that would normally have been used to deliver a traditional "cookbook" lab course can be simultaneously used to deliver an excellent learning experience and to enhance the faculty's research program. In addition, this course also ensures that every student has at least one research experience during their undergraduate career. If the student chooses to do additional research, Biology 2004 also helps that student be better prepared for participating in research projects in faculty labs across the campus. Students in Biology 2004 complete the entire progression of a research project, from exploring basic background information, through planning and conducting experiments, troubleshooting failures, and culminating in writing a scientific paper and delivering an oral presentation based on their results. Students meet weekly with an instructor,

but complete their project in an "open lab" format that allows great flexibility of work schedule. At the end of the first semester of the course, nearly 90% of the students reported that the research lab had met the goal of helping them experience authentic research. Eventually, approximately 225 students per semester will be enrolled in this course and selecting appropriate projects and developing strong models for supervision, training, and mentoring large numbers of students will therefore present substantial challenges. We will present a detailed description of the lab curriculum, describing how we are approaching these challenges based on the first research project, which focuses on exploring the evolution of cold adaptation in yeast.

1578/B29

Learning Scientific Content with Research and Reflection on Life and Death Issues.

K. K. Wentz-Hunter; Biological, Chemical and Physical Sciences, Roosevelt University, Chicago, IL

Cancer Biology [BIOL350/450] was a new course offered in the Spring 2008. In an effort to incorporate scientific content with social justice and creative writing, BIOL350/450 was designed with three major components. The first component provides students with knowledge of the fundamental principles of molecular and cellular biology of cancer cells. Cancer Biology was designed to illustrate basic aspects of cancer development, and to discuss how molecular genetic approaches can be used to reveal fundamental processes of carcinogenesis. The second component involves civic engagement and social justice. The students in the class were required to be actively involved in the formation of an American Cancer Society Colleges Against Cancer [CAC] student organization chapter at Roosevelt University. The third component was an activity in creative writing as well as research. Students were "given" symptoms of particular cancers and asked to research their symptoms, type of cancer, and treatment regimens from a variety of sources. In addition, students made decisions on their treatment options taking into consideration their insurance status and personal situations. Students maintained journals of their activities and were graded for accuracy of research and completion of objectives. The desired learning outcomes included: comprehension of the cellular and molecular aspects of cancer progression; evaluation of cancer risks and treatments; involvement in the Roosevelt University student community for cancer education and advocacy through the CAC; and appreciation of what cancer patients experience upon diagnosis and subsequent treatment via creative writing exercises in a journal through reflections and decision making.

Endoplasmic Reticulum (1578A – 1604)

1578A/B30

Investigating Whether Alanine-76 and Serine Phosphorylation Contribute to Alpha-Synuclein Aggregation, Membrane Association, and Toxicity in Yeasts.

M. Fiske, S. Valtierra, S. Herrera, M. White, M. Zorniak, S. DebBurman; Biology, Lake Forest College, Lake Forest, IL

Parkinson disease (PD) is a devastating and incurable neurodegenerative disorder that afflicts over one million Americans. The universal PD pathology is the presence of aggregated α-synuclein within dying midbrain substantia nigra neurons. The role these α-synuclein aggregates play in cell death is unclear, and whether these aggregates are neuroprotective or harmful to cells is still being debated. Here, we first tested the hypothesis that serine phosphorylation promotes α-synuclein aggregation and toxicity by studying two phosphorylation-deficient α-synuclein mutants (S87A & S129A) in budding and fission yeast models. In budding yeast, we found that both S87A & S129A altered alpha-synuclein localization within cells and, surprisingly, increased cellular toxicity, but to differing extents in different strains. In fission yeast, both S87A & S129A were also toxic, but to a milder extent. Secondly, we tested the hypothesis that alanine-76 (a key residue within the middle hydrophobic domain of α-synuclein) contributes to its aggregation and plasma membrane phospholipid association, by characterizing an A76E mutant in yeasts. In support of this hypothesis, in budding yeast, significantly less A76E localized to the plasma membrane. Furthermore, in fission yeast, less A76E was aggregated in live cells. In both yeasts, more A76E was found cytoplasmically diffuse. Our data support the notion that serine-87 and serine-129 phosphorylation may make alpha-synuclein cytoprotective and alanine-76 aids in alpha-synuclein aggregation and membrane phospholipid binding. (Supported by NSF-MRI, NSF-CCLI & NIH R15)

1579/B31

The Building of a Cellular Folding Compartment: a Holistic View of the ER Function Provided by Quantitative Genetic Interaction Maps.

M. C. Jonikas[4,1], S. R. Collins[4], E. Quan[4], J. Weibezahn[4], V. Schmid[5], V. Denic[4], B. Schwappach[5], P. Walter[1,3], J. Weissman[4,3], M. Schuldiner[2,4]; [1]Biochemistry and Biophysics, UC San Francisco, San Francisco, CA, [2]Molecular Genetics, Weizmann Institute of Science, Rehovot, Israel, [3]Howard Hughes Medical Institute, UC San Francisco, San Francisco, CA, [4]Cellular and Molecular Pharmacology, UC San Francisco, San Francisco, CA, [5]Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom

The endoplasmic (ER) provides a folding compartment responsible for the structural maturation of all secreted proteins. In order to support efficient folding, the ER maintains a highly specialized environment rich in chaperones and other folding factors, as well as a sophisticated quality control system that continually monitors its folding status and retains nonnative forms, while degrading terminally misfolded proteins. Additionally, the accumulation of misfolded proteins induces a stereotypical unfolded protein response (UPR) leading to the upregulation of hundreds of genes. While there has been intense efforts to study individual pathways that aid folding and correct misfolding, far less is known about how these various components work together to ensure proper folding. Here we have developed a novel approach, which allows us to systematically address this deficiency in our knowledge using the budding yeast S. cerevisiae as a test case. We first conducted a genome wide screen for proteins whose loss causes misfolding by quantifying UPR induction levels as a sensitive beacon of the ER folding status. This led to the identification of over 400 proteins, encompassing a remarkable array of different activities, that contribute to ER folding-far more than could be practically analyzed by traditional approaches. Accordingly, we developed a novel strategy based on systematic quantitative evaluation of folding defects in double mutants to determine how these factors function together to create a robust folding environment as well as to define the function for several uncharacterized and highly conserved complexes. More broadly, the two-

tiered quantitative genetic interaction approach described here provides a generalized strategy for dissecting complex cellular processes which should be readily adaptable to mammalian systems using RNAi technology.

1580/B32

Crystal Structure of the Tapasin/Erp57 Heterodimer and Insights into MHC Class I Peptide-Loading.

G. Dong[1,3], P. A. Wearsch[2], D. R. Peaper[2], H. Zheng[1], S. Mitchel[2], P. Cresswell[2], K. Reinisch[1]; [1]Department of Cell Biology, Yale University School of Medicine, New Haven, CT, [2]Department of Immunobiology, Howard Hughes Medical Institute, Yale University School of Medicine, New Haven, CT, [3]Max F. Perutz Laboratories, Vienna, Austria

Major Histocompatibility Complex (MHC) class I proteins bind endogenously derived peptides and present them at the cell surface for recognition by CD8+ T-cells, thus initiating specific immune responses against parasites and pathogens. Peptide binding takes place in the endoplasmic reticulum (ER) while the MHC class I molecule is sequestered within the peptide loading complex (PLC). The PLC both facilitates peptide loading and, in "peptide editing", ensures that only high affinity peptides are loaded. In addition to the MHC class I molecule, the PLC consists of the Transporter Associated with Antigen Processing (TAP), the lectin chaperone calreticulin, the protein disulfide isomerase ERp57, and a transmembrane glycoprotein, tapasin. TAP, ERp57, and tapasin form the core of the PLC. TAP transports peptides from the cytosol to the ER but is otherwise dispensible for peptide loading or editing. Tapasin is an MHC class I specific chaperone that in the PLC forms a disulfide-linked conjugate with ERp57. The tapasin/ERp57 complex recruits MHC class I and calreticulin to the PLC. To better understand PLC architecture and how tapasin/ERp57 functions within it, we have determined the structure of this heterodimer at a resolution of 2.6 Å. In ERp57, four thioredoxin-like domains *a*, *b*, *b'*, and *a'*, are arranged in a twisted U, whereas tapasin forms an L-shaped structure. The N terminus of tapasin consists of a β-barrel fused to an Ig-like fold, and the C terminus forms another Ig domain. The N-terminus of tapasin is bound between the a and a' domains of ERp57, with the two Cys-X-X-Cys catalytic motifs in ERp57 buried at the tapasin interface. We have identified a conserved surface on tapasin that is essential for peptide loading and MHC class I recruitment to the PLC, and we propose that within the PLC this surface stabilizes the MHC class I molecule in an open, peptide-receptive conformation. The structure provides a first view of two ER resident proteins interacting in a chaperone complex and, since tapasin is a substrate of ERp57, a first view of a PDI family member in complex with protein substrate.

1581/B33

Autocrine Motility Factor (AMF)/Phosphoglucose Isomerase (PGI) Regulates Ca2+ Coupling and Interaction between Smooth Domains of Endoplasmic Reticulum and Mitochondria.

M. Fu, P. St-Pierre, I. R. Nabi; Cellular and Physiological sciences, University of British Columbia, Vancouver, BC, Canada

The glycolytic enzyme phosphoglucose isomerase (PGI), or autocrine motility factor (AMF), is an extracellular ligand for the glycoprotein 78/autocrine motility factor receptor (gp78/AMFR). Both PGI/AMF and gp78/AMFR are implicated in tumor progression and PGI/AMF has been shown to promote cell survival. Previous studies showed that the 3F3A monoclonal antibody specific to gp78/AMFR labels mitochondria-associated smooth endoplasmic reticulum (ER) tubules and that this association is regulated by free cytosolic Ca2+. ER-mitochondria interaction is central to regulation of cellular Ca2+ homeostasis as well as cell death and survival. Thus, this study investigated the effect of AMF on ER-mitochondria association and intracellular Ca2+ dynamics. PGI/AMF stimulation of Cos7 cells up-regulates AMFR expression and causes dissociation between 3F3A-labeled smooth ER tubules and mitochondria. AMF stimulation decreases both agonist- and thapsigargin- evoked Ca2+ release from ER as well as Ca2+ uptake by mitochondria, but does not affect basal cytosolic Ca2+ levels, Ca2+ entry or Ca2+ reuptake by ER. PGI/AMF stimulation therefore mainly influences Ca2+ coupling between ER and mitochondria. These results suggest that by impacting on ER-mitochondria association, AMF may regulate intracellular Ca2+ homeostasis and thereby influence tumor cell survival. This project is supported by the Canadian Institutes for Health Research.

1582/B34

Maintaining ER Homeostasis: a Genome-Wide Analysis Identifies Novel Components Involved in Protein Folding, Glycosylation and Trafficking.

E. Miller[1], A. Copic[1], M. Dorrington[1], s. pagant[1], M. Lee[2], J. Barry[1]; [1]Biological Sciences, Columbia University, New York, NY, [2]Microbiology, Columbia University, New York, NY

The endoplasmic reticulum (ER) must maintain a delicate balance of efficiently retaining resident proteins while managing a diverse cast of client proteins that are acted upon by those residents before being selected for forward transit through the secretory pathway. We devised a genome-wide screen for yeast mutants that secrete significant amounts of the ER resident chaperone, Kar2p, a defect that may indicate a breakdown in ER homeostasis. We identified 72 mutants with significant ER retention defects and analyzed these strains with respect to secretory protein biogenesis, induction of the unfolded protein response, ER quality control and the fidelity of vesicle traffic. We identified 21 mutants with defects in protein glycosylation and/or maturation of GPI-anchored proteins, including 10 components with previously uncharacterized functions. 19 mutants secreted Kar2p dependent on the ability to mount an unfolded protein response, consistent with roles for these components in protein folding and degradation. Several mutants showed aberrant localization of the Kar2p retrograde receptor, Erd2p, suggesting that secretion of Kar2p in these mutants results from inefficient retrieval of escaped Kar2p from the Golgi. Finally, 15 mutants showed increased Golgi modification of a model misfolded protein, suggesting ER quality control is perturbed in these strains. A significant number of these mutants also shared a bypass-of-sec-thirteen (BST) phenotype, whereby the otherwise essential vesicle coat component, Sec13p, is dispensable. We propose a novel mechanism that permits these cells to survive in the absence of a key structural element of the vesicle trafficking machinery: alterations in the physical properties of the lipid bilayer in these mutants may create a more malleable membrane that can be deformed into a highly curved transport vesicle in the absence of the rigidifying effect of Sec13p. Together, our collection of mutants shed significant insight into many components that contribute to ER homeostasis through a variety of cellular processes.

1583/B35

The Influence of Tapasin on Peptide Binding by MHC Class I Molecules.

P. Pudiavettil Tayakuniyil[1], M. Zacharias[2], S. Springer[1]; [1]Biochemistry and cell biology, Jacobs University Bremen, Bremen, Germany, [2]Bioinformatics and Computational Biology, Jacobs University Bremen, Bremen, Germany

Major histocompatibility complex (MHC) class I molecules bind intracellular peptide ligands in the endoplasmic reticulum (ER) and present them at the cell surface to cytotoxic T lymphocytes. Since peptide-empty class I molecules at the cell surface would be inefficient and probably dangerous for cells, class I molecules must select peptides with dissociation rates that are sufficiently slow to allow survival of the peptide-class I complex during the transit to the cell surface and for some time thereafter. Elliott and collaborators have shown that class I molecules, however, first bind low-affinity peptides in the ER and later exchange them for high-affinity ones before leaving for the Golgi apparatus (Williams et al., Immunity 16 (2002) p. 509). This process, termed peptide editing, depends on the class I - specific assembly factor, tapasin, which binds directly to class I molecules in the ER. How does tapasin work? Based on our molecular dynamics simulations (Zacharias and Springer, Biophys. J. 87 (2004), p. 2203), we hypothesize that it may bind to and deform the peptide binding site of class I molecules by moving one flexible helix away from the peptide. As a consequence, some of the hydrogen bonds between the class I molecule and the peptide backbone may break and reduce the affinity between peptide and class I molecule. This would lead to a rapid dissociation of low-affinity peptides from the class I molecule. As a conclusion, this hypothesis predicts that tapasin universally decreases the affinity of class I molecules for peptide ligands. Since it is difficult to recreate the entire class I loading complex with purified proteins (it contains several other chaperones), we investigating peptide binding to class I molecules in the lumen of isolated microsomal membranes in the presence and absence of tapasin. Class I molecules are transcribed, translated, and translocated in vitro. Peptide is then added to the microsomes, and peptide-class I complexes precipitated by conformation-specific antibodies. We have observed that tapasin mediates the binding of high affinity peptides to MHC class I molecules in the ER.

1584/B36

Role of Reticulons and DP1/YOP1P in Maintaining ER Tubules.

C. Voss[1], Y. Shibata[2], T. A. Rapoport[2], G. K. Voeltz[3], W. A. Prinz[1]; [1]LCBB,NIDDK, NIH, Bethesda, MD, [2]Department of Cell Biology, Harvard Medical School/HHMI, Boston, ME, [3]DMCDB, University of Colorado-Boulder, Boulder, CO

The endoplasmic reticulum (ER) forms a highly dynamic network of tubules. In the yeast S. cerevisiae, the tubular portions of the ER are mostly near the cell periphery while the perinuclear ER is sheet-like. Recently we found that a family of membrane proteins required to maintain the tubular structure of the ER, the reticulons (Rtn1p, Rtn2p) and reticulon-like proteins (DP1/Yop1p), are localized exclusively in the peripheral ER. Our lab is interested in how these proteins shape the ER in S. cerevisiae. We found that these proteins must oligomerize to stabilize membrane tubules. To better understand the mechanism of their localization, we isolated mutations in Rtn1p that are distributed throughout the entire ER. We demonstrated that these mutations decrease the ability of Rtn1p to oligomerize, suggesting that the oligomerization of Rtn1p with itself or with other proteins is important to keep the protein in the highly curved membranes of the ER tubules. Interestingly, the mobility of Rtn1p within the ER membrane is affected by ATP-depletion, suggesting that Rtn1p oligomerization must be highly regulated. We are currently looking at the role of various kinases in regulating Rtn1p. We would also like to understand why cells need tubular ER since cells lacking the reticulons and YOP1 have no apparent phenotype despite the absence of tubular ER. We identified mutations in genes that are synthetically lethal with deletion of RTN1, RTN2 and YOP1. Interestingly, two of these have defects in the same lipid biosynthesis pathway. Loss of these proteins leads to overproduction of phospholipids, which affects ER structure. These findings suggest that the phospholipids level in the ER and reticulons are required for proper ER function.

1585/B37

Mechanisms of Cellular Protein-Tyrosine Phosphatase 1B-Substrate Interaction.

F. G. Haj[1], S. Wimmer-Kleikamp[2], V. Roukos[3], A. Kinkhabwala[4], B. Neel[5], P. Bastiaens[4]; [1]Nutrition, University of California Davis, Davis, CA, [2]University of Sydney, Sydney, NSW, Australia, [3]Universiry of Patras, Patras, Greece, [4]Max Planck Institute of Molecular Physiology, Dortmund, Germany, [5]Ontario Cancer Institute, Toronto, ON, Canada

Protein-tyrosine phosphatase 1B (PTP1B) is a ubiquitously expressed non-receptor PTP that plays an important role in regulating diverse cell signaling pathways, including those initiated by several receptor tyrosine kinases (RTKs) and cytokine receptors, as well as integrins and cadherins. PTP1B is tightly anchored to the cytoplasmic face of the endoplasmic reticulum (ER) via a hydrophobic C-terminal targeting sequence, which constrains its accessibility to key substrates. Previously, we and others showed that PTP1B dephosphorylates RTKs such as the epidermal growth factor receptor (EGFR) and insulin receptor (IR) after receptor endocytosis, presumably after they transit past the ER. How PTP1B accesses plasma membrane (PM)-bound substrates that do not endocytose has remained unclear. To delineate the mechanism of PTP1B interaction with such substrates, we utilized advanced quantitative cellular imaging such as photoactivation and fluorescence lifetime imaging microscopy in combination with substrate-trapping approaches. Our findings indicate that the ER extends outwards to be in close proximity to the PM only at apparently specialized regions of cell-cell contact, enabling PTP1B to dephosphorylate key substrates at these sites. In addition, the dynamics of PTP1B interaction with novel substrates was assessed. These studies show that ER-anchored PTP1B plays an important role in regulating signaling at regions of cell-cell contact and identify PM proximal sub-regions of the ER as an important site for regulating cellular signaling events.

1586/B38

Mutant Huntingtin Inhibits gp78 Interactions with Polyubiquitin and p97/VCP but Is Targeted for Degradation by gp78.

H. Yang[1], Y. Zhong[1], S. Luo[2], P. Lim[1], C. Liu[1], M. J. Monteiro[1], S. Fang[1]; [1]UMBI, Baltimore, MD, [2]Cambridge Institute for Medical Research, Cambridge CB2 2XY, United Kingdom

Huntington's disease (HD) is a dominant genetic neurodegenerative disease caused by polyglutamine (polyQ) expansion in the N-terminal portion of huntingtin (htt) protein. Accumulation of polyQ-expanded N-terminal fragment of htt is toxic to neurons and appears to be an obligate step for HD pathogenesis. Endoplasmic reticulum (ER) stress induced by the toxic fragment is an important mechanism underlying

neuronal degeneration. Recent studies indicate that htt is localized to the membrane of ER, suggesting that mutant htt may directly interfere with ER function and induces ER stress. To explore this mechanism, we identified gp78, an ER-anchored RING finger E3 ubiquitin ligase, as a novel htt-interacting protein. Using htt fragments: amino acids 1-588 with 17Q or 138Q (httN17Q or httN138Q), as wild-type (wt) and toxic httN, we demonstrated that wt httN negatively regulates gp78 E3 activity by interacting with the cue domain of gp78, thus, inhibiting gp78 binding to polyubiquitin. Wt httN interacts with the gp78-p97/VCP complex. Toxic httN aggravates the inhibition of gp78 E3 activity by disrupting gp78-p97/VCP interaction and by its higher affinity to gp78 than wt httN. On the other hand, gp78 ubiquitinates and targets httN138Q but not httN17Q for degradation. Surprisingly, gp78-mediated degradation of toxic httN does not require gp78 interactions with polyubiquitin and p97/VCP although both of which are essential for gp78-mediated degradation of misfolded ER transmembrane and luminal proteins. Our results indicate that while toxic httN impairs gp78's ability to remove misfolded proteins from the ER and induces ER stress, gp78 can counteract the toxicity of mutant httN by targeting it for degradation. Therefore, the interplay between toxic httN and gp78 may play an important role in the progression of HD (Supported by grants from the National Institutes of Health R01 GM69967 to SF and GM066287 to MJM).

1587/B39

Intrinsic Structural Sensors Report Glycoprotein Folding Failures by Displaying Bipartite Degradation Signals.

W. Xie[1,2], K. Kanehara[1], D. Ng[1,2]; [1]Temasek Life Sciences Laboratory, Singapore, Singapore, [2]Department of Biological Sciences, National University of Singapore, Singapore, Singapore

Endoplasmic reticulum (ER) quality control mechanisms monitor the folding of nascent polypeptides of the secretory pathway. These are dynamic processes that retain folding proteins, promote the transport of conformationally mature proteins, and target misfolded proteins to ER associated degradation (ERAD) pathways. Aided by the identification of numerous ERAD factors, late functions that include substrate extraction, ubiquitination, and degradation are fairly well described. By contrast, the mechanism of substrate recognition and the molecular logic used to differentiate folding, folded, and misfolded proteins remain mysterious. For some substrates, a specific N-linked glycan forms part of the recognition code but how it is read is unclear. In this study, systematic analysis of model substrates revealed such glycans mark structural determinants that are sensitive to the overall folding state of the molecule. Normally, these segments fold into the mature structure to pass the ERAD checkpoint. However, should a molecule fail to fold completely, they form a bipartite signal that comprises the unfolded local structure and enzymatically modified N-linked glycan. Only if both elements are present will the substrate be targeted to the ERAD pathway for degradation.

1588/B40

PACS-2 Targets Calnexin to the Mitochondria-Associated Membrane and Redistributes It within the ER during Apoptosis Onset.

E. M. Lynes, N. Myhill, J. Nanji, T. Simmen; Cell Biology, University of Alberta, Edmonton, AB, Canada

PACS-2 is an intracellular sorting protein that sorts proteins at the level of endosomes, mitochondria and the endoplasmic reticulum (ER). Our recent results have shown that PACS-2 also regulates the distribution of the ER chaperone calnexin between domains of the ER and the plasma membrane. Besides its well-established function as a chaperone, calnexin also interacts with ER calcium pumps that localize to the mitochondria-associated membrane (MAM). Depending on ER homeostasis, varying amounts of calnexin target to the plasma membrane. Our results describe how the interaction of calnexin with the cytosolic sorting protein PACS-2 distributes calnexin between the rough ER, the MAM and the plasma membrane. Under control conditions, more than 80% of calnexin localizes to the ER with the majority on the MAM. PACS-2 knockdown disrupts the calnexin distribution within the ER and increases its levels on the cell surface. Furthermore, PACS-2 and calnexin form a complex following extensive ER stress that coincides with the induction of the ER stress gene CHOP. PACS-2/calnexin complex formation is preceded by a temporary interaction of calnexin with the initiator caspase-8. The PACS-2/calnexin complex subsequently shifts away from heavy membranes (rough ER and MAM) and makes the calnexin interactor BAP31 available for caspase degradation. Our results thus describe a novel mechanism that promotes ER-stress derived apoptosis.

1589/B41

Genetics, Genomics and Cell Biological Approaches to the Regulation of Lipid Droplet Size in C. Elegans.

S. Zhang; Stowers Institute, Kansas City, MO

Shaobing Zhang, Andrew Box, Ningyi Xu, Fengli Guo, & Ho Yi Mak Stowers Institute for Medical Research, 1000 E. 50th Street, Kansas City, MO 64110 Lipid droplet emerges as a specialized cell organelle in which triacyglycerol (TAG, fat) is stored. Increased lipid droplet size in adipose tissues is associated with excess fat storage and obesity. In order to understand how the size of lipid droplets is regulated, we isolated *C. elegans* mutants that accumulate bigger intracellular lipid droplets. The big lipid droplet phenotype is associated with altered TAG and cholesterol ester levels and is diet-dependent. Cloning of three mutants from our collection revealed the involvement of a fatty acid beta-oxidization pathway in the regulation of lipid droplet formation. Fatty acid compositions of mutant vs. wild type worms and of different diets were thus compared. We carried out cell biological and electron microscopic studies to characterize the big lipid droplets. A hypothesis on the formation of big lipid droplets will be presented.

1590/B42

IRE-1 and HSP-4 Play Key Roles in Energy Homeostasis via Novel Fasting-Induced Lipases in C. Elegans .

H. Jo*, J. Shim*, J. Lee, J. Huh, J. Lee#, J. Kim#; Department of Biological Sciences, Seoul National University, Seoul, South Korea

The endoplasmic reticulum (ER) is an organelle associated with lipid metabolism. However, the involvement of the ER in nutritional status-dependent energy homeostasis is largely unknown. Here we show that IRE-1, an ER protein involved in the unfolded protein response, and HSP-4, an ER chaperone, regulate the expression of novel adipose triglyceride lipase (ATGL)-related fasting-induced lipases, FIL-1 and FIL-2, for fat granule hydrolysis upon fasting in *C. elegans*. Interestingly, failure of ire-1 and hsp-4 mutant animals to hydrolyze intestinal fat granules during starvation impaired their motility, whereas glucose supplementation rescued the motility defect, implicating the importance of ire-1/hsp-4-dependent lipid homeostasis for energy supply from stored fat droplets during fasting. Taken together, our data suggest that the

ER resident proteins IRE-1 and HSP-4 are key nutritional sensors that modulate expression of inducible lipases to maintain energy homeostasis.

1591/B43

Positive Cooperativity of the P97 AAA Atpase Is Critical for Cell Viability.

M. Esaki, S. Nishikori, K. Yamanaka, T. Ogura; Institute of Molecular Embryology and Genetics, Kumamoto University, Kumamoto, Japan

p97 (Cdc48p in yeast) is an abundant essential protein of the family of ATPases associated with diverse cellular activities (AAA). p97 is well known to participate in various cellular processes including ER-associated protein degradation, reorganization of the Golgi complex, and cell cycle control. p97 is composed of an N-terminal domain, which binds to co-factors, followed by two AAA ATPase domains (D1 and D2), which form a tandem ring-shaped hexamer. *In vitro* analyses have suggested that the D1 AAA domain is responsible for the oligomerization whereas the D2 AAA domain mediates the major ATPase activity. Using the yeast *Saccharomyces cerevisiae*, we have asked whether the ATPase activity of each AAA domain is required for essential functions of Cdc48p *In Vivo*. Mutations in any residues responsible for ATP binding or ATP hydrolysis of the D2 domain led to cell death. In contrast, most mutants with deficiency in ATP binding or ATP hydrolysis of the D1 domain looked healthy, indicating that the ATPase activity of the D2 domain, but not of the D1 domain, is essential for cell viability. However, exceptionally lethal was a substitution of the conserved catalytic glutamate residue in the D1 domain with glutamine (E-Q mutation), which fails to activate a water molecule attacking on the γ-phosphate of bound ATP. Why does this particular mutation in the non-essential D1 domain compromise essential functions of Cdc48p? *In vitro* ATPase analyses showed that the D2 domain exhibits positive cooperativity. The E-Q substitution of the D1 domain not only affected the ATPase activity of the D1 domain but also lowered that of the D2 domain, and most interestingly led to lose the positive cooperativity of the D2 ATPase cycle. We isolated intragenic suppressors of the E-Q mutant, which allow robust cell growth at 30°C. These suppressor mutants re-exhibited the positive cooperativity and showed a good correlation between the cell growth and the positive cooperativity of the ATPase cycle in the D2 domain. Taken together, we propose that the ATPase activity itself of the D1 domain is dispensable but regulates the ATPase cycle of the D2 domain, whose positive cooperativity is crucial for the essential function of p97.

1592/B44

Arabidopsis Prenylated Rab Acceptor 1 Localizes to the ER and Golgi Complex and Differentially Regulates ER Exit of Proteins Destined for Golgi and Post-Golgi Compartments.

M. Lee, C. Jung, H. Byun, M. Min, I. Hwang; POSTECH, Pohang, South Korea

Prenylated rab acceptors (PRAs), which belong to the Ypt-interacting protein (Yip) family of small membrane proteins that bind prenylated Rabs, are thought to aid targeting of Rabs to their respective endomembrane compartments. Here, we investigated the biological role of the Arabidopsis thaliana prenylated Rab acceptor 1 (AtPRA1), which is homologous to animal prenylated rab acceptor 1 (PRA1) and yeast Yip3p. Subcellular fractionation experiments demonstrated that AtPRA1 is an integral membrane protein. Colocalization with various organellar markers demonstrated that AtPRA1 localizes primarily to the Golgi complex with a minor fraction in the ER. AtPRA1 exhibits differing affinities for multiple Rab proteins, including strong binding to AtRabD2a and AtRabA5c, but no binding to AtRabF1. In addition, AtPRA1 undergoes homotypic interactions, as well as heterotypic interactions with closely related, but not distantly related, AtPRA isoforms. Overexpression of HA:AtPRA1 inhibits trafficking of sporamin:GFP to the central vacuole and secretion of invertase:GFP into the medium and causes these proteins to accumulate in the ER. In addition, HA:AtPRA1 blocks recycling of an ER retrieval motif-containing membrane protein between the ER and Golgi complex. However, overexpression of HA:AtPRA1 does not inhibit trafficking of ST:GFP or Kam1(dC):mRFP, which are Golgi resident proteins. Based on these results, we propose that AtPRA1 differentially regulates the ER exit of cargo proteins based on their destinations.

1593/B45

Effects of Various ER Stress on Activated Macrophage.

H. Kim, S. Kim, D. Lee; Life Sciences, University of Seoul, Seoul, South Korea

The endoplasmic reticulum(ER) exerts multiple cellular function. Recent studies suggest that ER stress and unfolded protein response (UPR) play a significant pathological role in inflammation encompassing immune response, diabetes, and tumor development. Inflammatory pathways can be initiated by extracellular signal from cytokines and excess of lipids or by intracellular stress caused by ER stress agents. ER stress leads to activation of inflammatory signaling pathway employing JNK and IKK. These pathway lead to the production of additional inflammatory mediators through transcriptional regulation. Macrophages are phagocytes, acting in both non-specific defense (or innate immunity) as well as to help initiate specific defense mechanisms (or cell-mediated immunity). The present study is to characterize the effect of ER stress on the differentiating macrophage (Raw264.7). ER stress was caused by three agents of ER stress: tunicamycin (inhibitor of protein N-glycosylation), brefaldin A (BFA), and thapsigargin. BFA can block protein transport from ER to Golgi and Thapsigargin lowers calcium concentration with ER. When treated tunicamycin, macrophage cell, activated by pretreated lipopolysaccharide (LPS), leads to increased expression of inflammatory cytokines of IL6 and TNFα. Under treatment with BFA or thapsigargin, activated macrophage show minimal expression of inflammatory cytokines different from that of tunicamycin. These two inconsistent results strongly imply that ER stress exert a multiplity of outcomes depending on the cause of such stress. Macrophage might responds differently to the corresponding level of ER stress and UPR within ER. (This study was supported by the 2008 BioGreen21 from KRDA).

1594/B46

Anti-Apoptotic Role of GRP94 during Adipogenesis Under Endoplasmic Reticulum Stress.

S. Kim, H. Kim, D. Lee; Life Sciences, University of Seoul, Seoul, South Korea

Excessive lipid deposition in endoplasmic reticulum (ER) causes adipocytes to undergo cellular stress. This adipocyte stress accompanies typical endoplasmic reticulum stress (ERS) characterized by unfolded protein response and other pathological outcomes. Despite progression in ERS, however, adipocytes rarely proceed to apoptosis. The resistance to apoptosis is widely perceived as the main cause of adipocytes

leading to obesity. This study is to characterize a potential mechanism whereby adipocyte avoid apoptosis to sustain pathological state of obesity. This study utilizes induction of ERS on differentiating preadipocytes of 3T3-L1. When 3T3-L1 preadipocytes was induced for adipogenesis, they showed typical ERS, which increased after treatment with tunicamycin. However, differentiated 3T3-L1 hardly proceed to apoptosis based on the assays of caspase 12 and FACS analysis. This is very different from preadipocytes and C2C12, the myogenic counterpart of 3T3-L1, which showed apoptosis. In addition, GRP94 expression was significantly decreased under tunicamycin treatment compared to preadipocytes and C2C-12. In conclusion, adipocytes may have an antiapototic mechanism in the presence of strong ERS and the increased level of GRP94 may serve as a key role whereby adipocytes reach to the obesity circumventing the apoptotic barrier. (This study was supported by the 2008 BioGreen21 from KRDA)

1595/B47

Differential Effects of Induced Stress on an Erp29 Deficient Cell Line.

R. Jacob[1], I. Hirsch[1], D. Diao[1], N. V. Harpham[1], H. Urlaub[2], D. M. Ferrari[1]; [1]Independent Junior Research Group, Max Planck Research Unit for Enzymology of Protein Folding, Halle (Saale), Germany, [2]Bioanalytical Mass Spectrometry Group, Max Planck Institute for Biophysical Chemistry, Göttingen, Germany

We have developed the first viable knockout model for a Protein Disulfide Isomerase (PDI)-related protein, ERp29, and we have employed this model to investigate the role of ERp29 and other family members in protein secretion and their function during stress signalling in the endoplasmic reticulum (ER). Using primary embryonal fibroblast cell lines established from ERp29 deficient and wild-type mice, the effects of ERp29 deficiency and the response of ERp29 deficient cells to stress inducing agents (hydrogen peroxide, Tunicamycin) was studied. Levels of ER and cytosolic chaperones as well as components of stress response pathways were examined by Western blotting. Additionally 2D-DIGE (Difference Gel Electrophoresis) technology, together with LC-MS, was applied to monitor small changes in protein levels in full cell lysates. Among the differentially regulated spots, we identified stress proteins, metabolic enzymes and as yet uncharacterized proteins. Using this approach, we have begun to characterize the physiological roles of ERp29. Overall, our results provide a valuable insight into the multi-facetted effects of PDI-related protein regulation in the cell.

1596/B48

Analysis of Peptide Binding Properties of PDI-Related Proteins.

A. Funkner[1], N. N. Barak[1], M. Schutkowski[1], L. W. Ruddock[2], D. M. Ferrari[1]; [1]Independent Junior Research Group, Max Planck Research Unit for Enzymology of Protein Folding, Halle (Saale), Germany, [2]Department of Biochemistry, University of Oulu, Oulu, Finland

Protein disulfide isomerase (PDI)-related proteins are ER-resident proteins which act as oxidoreductases and/or exhibit chaperone functions. Up to now at least 19 different members of the human PDI family have been identified or predicted to exist. Despite differences in amino acid composition they share at least one structurally similar domain containing a thioredoxin (thx) fold. Members of the PDI-D subfamily (e.g. ERp29) are composed of a thx-like domain and a C-terminal all helical D-domain. Here, we show that PDI-related proteins display differential binding properties in immobilized protein interaction studies. In pull down assays using microsomal extracts, ERp29 and other PDI-related proteins interact with different secretory proteins. Using peptide micro-array chip screening techniques, we find that PDI-related proteins bind model peptides containing discrete recognition motifs defined by aromatic residues. Importantly, disruption of these motifs leads to loss of binding. Although the exact nature of interacting motifs is likely to require further clarification in most cases, certain similarities can nevertheless be discerned. In PDI-D proteins, we find that whereas the b-domain is sufficient for peptide binding, the D-domain may be involved in binding to larger proteins. Furthermore, for ERp29 and other PDI-related proteins, peptide binding is pH and ionic strength dependent. Our data is in agreement with the existence of a discrete binding site in the b' domain of PDI proteins, and provides a comparative overview of common binding motifs for PDI-related proteins. In conclusion, although peptide binding can occur to similar peptides, protein selectivity differs between the various PDI-related proteins.

1597/B49

Discrepancies in Genetic Screens of Different Sacchromyces Cereviseae Deletion Collections for Cold Sensitivity.

A. Felthauser, K. Braue, D. Schaleben-Boateng, R. Wright; Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN

A subset of ER-associated degradation (ERAD) genes, UBC7, CUE1 and DOA10, are required for growth of yeast at lower temperatures (<10oC; Loertscher, et. al. 2006, Eukaryotic Cell). To further explore the physiology and genetics of growth at low temperatures, we took advantage of two collections of Sacchromyces cereviseae deletion mutants. Specifically, we screened 4741 homozygous diploid deletion mutants (Open Biosystems, Huntsville, AL) and 4774 haploid deletion mutants (Invitrogen, Carlsbad, CA). Within these collections, we identified 558 genes that were required for growth at 10oC. However, only 107 of these genes were identified in both the haploid and homozygous diploid sets. In fact, the majority of the genes (434) produced discrepant cold-sensitive phenotypes in the two deletion collections. The discrepancy may reflect inaccurate strain curation or the presence of cryptic mutations. To test these hypotheses, we are currently attempting to complement the cold-sensitive genes using plasmids from the Yeast Genomic Tiling Collection (Open Biosystems, Huntsville, AL). These results emphasize the need to verify the identity of the genes obtained by analysis of deletion collection screens.

1598/B50

Aurintricarboxylic Acid Increases the Permeability of the Endoplasmic Reticulum to Small Molecules.

W. Wonderlin; Biochemistry, West Virginia University, Morgantown, WV

Aurintricarboxylic acid (ATA) is a polyanionic, polyaromatic drug that interferes with the binding of ribonucleic acids to proteins. ATA is known to stimulate the release of ribosomes bound to the endoplasmic reticulum (ER), perhaps by competing with ribosomal RNA for the same binding site(s) on the Sec61 translocon. We recently reported (Pflugers Arch., 2008) that the binding of ribosomes to Sec61 translocons increased the permeability of the ER to a small polar solute, 4-methyl-umbelliferyl-alpha-D-glucopyranoside (4MG). The putative binding of ATA and ribosomes to the same site(s) on the translocon led us to hypothesize that the binding of ATA to ribosome-free translocons might

mimic the binding of ribosomes and also increase the permeability of the ER to 4MG. We report here that ATA increased the permeability of ribosome-free ER microsomes prepared from pig pancreas, with half-maximal stimulation near 10 μM and a magnitude comparable to the stimulatory effect of ribosomes. The stimulatory effect of ATA was only observed after the ribosomes had been removed by EDTA. The non-additive, but equivalent, stimulatory effects of ribosomes and ATA were consistent with competition between ATA and ribosomes for binding to translocons. ATA also produced a dose-dependent increase in the permeability of the ribosome-free, intact ER in CHO-K1 cells. The stimulatory effect of ATA was not mimicked by other polyanions, including polyglutamate, Dextran Blue, and EDTA, or by salicylic acid and 2,4-dihydroxybenzoic acid, which are structurally similar to the aromatic acid moieties of ATA. Commercially-available ATA is a polydisperse polymer. Polymers larger than 3500 daltons accounted for 63% of the total ATA and 80% of its stimulatory effect on the ER. In summary, polymeric ATA mimicked the binding of ribosomes in its ability to increase the permeability of the ER. Therefore, ATA might serve as a useful pharmacological tool for studying how the permeability of the Sec61 translocon is regulated.

1599/B51

hsp90 Is Required for Dislocation of the Cholera Toxin A1 Polypeptide from the Endoplasmic Reticulum to the Cytosol.

M. Taylor, K. Teter; Molecular Biology and Microbiology, University of Central Florida, Orlando, FL

Cholera toxin (CT) is an AB5 toxin that moves from the cell surface to the endoplasmic reticulum (ER) by retrograde vesicular transport. In the ER, the catalytic A1 polypeptide dissociates from the rest of the toxin and enters the cytosol by exploiting the quality control system of ER-associated degradation (ERAD). The driving force for CTA1 dislocation into the cytosol is unknown. Here, we report that hsp90 activity is required for CTA1 passage into the cytosol. Experiments using surface plasmon resonance demonstrated that hsp90 binds to CTA1 in an ATP-dependent manner that is blocked by geldanamycin, an established hsp90 inhibitor. CT activity against cultured cells was also blocked by geldanamycin, as was the ER-to-cytosol export of CTA1. This work thus establishes a functional role for hsp90 in the extraction of a soluble ERAD substrate from the ER.

1600/B52

An Experimental Model of Inducible Lipid Droplet Assembly in Yeast.

P. Mardones, T. C. Walther; Organelle Architecture and Dynamics, Max Planck Institute of Biochemistry, Martinsried, Germany

Lipid droplets (LD) have recently been recognized as highly dynamic organelles that modulate and coordinate important cellular functions, most notably participating in energy homeostasis. Despite great progress in the understanding of the cell biology of LDs, the detailed mechanism of LD biogenesis is still a matter of speculation and debate. To characterize the LD biogenesis process, we have generated yeast strains that allow the induction of LD formation in a strain background that otherwise has no LDs. We achieved this by replacing the endogenous promoters of the enzymes catalyzing the last committed steps of neutral lipid synthesis (Dga1, Lro1, Are1, Are2) with the inducible GAL1/10 promoter. Under repressing conditions, these strains do not express any of the neutral lipid synthesizing enzymes and do not generate LDs. In contrast, galactose induces the expression of these enzymes, which leads to increased levels of triacylglycerol and sterol esters, with concomitant formation of LDs. This inducible system allows us to characterize LD formation in detail using a combination of LD proteomics, lipidomics, electron microscopy and time lapse microscopy.

1601/B53

Functional Characterization of Domains within the ER-Resident, Lipid Raft Associated Proteins erlin-1 and -2.

M. B. Hoegg, D. T. Browman, M. E. Resek, S. M. Robbins; Southern Alberta Cancer Research Institute, Dpt. of Biochemistry and Molecular Biology, University of Calgary, Calgary, AB, Canada

Using an antibody screen against isolated lipid raft proteins from human myelomonocytic cells, our laboratory has recently discovered two novel highly related proteins. Based on their enrichment in detergent resistant membrane fractions (DRMs) and their localization to the endoplasmic reticulum (ER) they were named erlin-1 and -2 (ER Lipid Raft Protein -1 and -2). The function of the erlin proteins is still relatively unexplored, but a recent study suggests that erlin-2 might act as a substrate recognition factor in ER associated protein degradation (ERAD).The N-terminal and central regions of the erlins are occupied by a large domain called Stomatin/ Prohibitin/ Flotillin/ HflK/C (SPFH) domain, which they share with a number of other proteins. Members of the group of SPFH domain containing proteins differ in respect to subcellular distribution and function, but all of them are associated with DRMs and form oligomers, suggesting that these properties are important for their function. The objective of this study is to characterize oligomerization of the erlins and to identify regions within these proteins which mediate oligomerization and DRM association. We have found that erlins form homo- and hetero-oligomers with each other and are also part of high molecular weight (MW) protein complexes. Regions within the erlins downstream of the SPFH domain were found to be required for DRM association and oligomerization. Furthermore, mutation of a single phenylalanine within a stretch of hydrophobic amino acids close to the C-terminus (erlin F305A), prevents the protein from associating with high MW complexes. The erlin F305A mutant is enriched in DRMs and oligomerizes with wild-type erlin. This suggests that oligomerization and high MW complex formation are distinct biochemical properties of the erlins, and oligomerization and DRM association are not dependent on high MW complex formation. Ectopic expression of the erlin F305A mutant prevents endogenous erlin proteins from association with high molecular weight complexes, suggesting that it could act as a dominant negative mutant. The data presented here provide important tools to study erlin function in the ER.

1602/B54

Regulation of Cell Death by a Novel ER Protein.

J. Wu, Y. Tang, H. Li; Children's Memorial Research Center, Chicago, IL

The endoplasmic reticulum (ER) is a membrane network that plays a central role in lipid and protein synthesis and Ca2+ signaling. Accumulation of misfolded proteins in the ER lumen induces a coordinated cellular response known as the ER stress response or unfolded protein response (UPR). UPR is involved in the pathogenesis of many human diseases, including neurodegenerative diseases, diabetes and certain liver and heart diseases. We report here the characterization of a novel ER stress-induced protein C20orf116 as an important regulator

of cell death. C53/LZAP is a putative tumor suppressor that regulates NF-kB signaling and DNA damage response. In attempt to isolate C53 interactors, we used C53 as the bait and performed immunoprecipitation and mass spectrometry. As a result, we identified C20orf116 as a novel C53-interacting protein. C20orf116 is a highly conserved protein whose orthologs can be found in vertebrates, invertebrates and plants, but not in yeast and bacteria. Human C20orf116 contains 314 amino acid residues with a signal peptide and a PCI domain that is frequently found in the subunits of the proteasome, COP-9-complex and translation initiation factors. C20orf116 is expressed in multiple tissues and cancer cell lines. Further, immunofluorescence (IF), electron microscope (EM) and subcellular fractionation assays showed that C20orf116 is localized at the ER in multiple cell lines. Ectopic expression of C20orf116 multiple cell lines led to accumulation of organized smooth ER (OSER). Interestingly, we found that C20orf116 expression was significantly elevated under ER stresses that were induced by Ca2+ pump inhibitor thapsigargin (TG) and glycosylation inhibitor tunicamycin (TM). Importantly, overexpresion of C20orf116 promoted cell death induced by various death stimuli, while siRNA-mediated knockdown significantly inhibited the cell death. Taken together, our results suggest that C20orf116 may be is a novel ER protein that plays pivotal roles in regulation of the ER dynamics and cell death signaling.

1603/B55

Life in the Cold: Characterization of Ten Psychrophilic Yeast Species.

Z. Fortier[1], L. Grengs[1], M. von Hohenberg[1], M. Kanneganti[1], K. Kwong[1], S. Lin[1], X. Lou[1], E. Myhre[1], K. Petersen[1], J. Sarapas[1], N. Talledge[1], K. Zimmerman[1], A. Felthauser[1], M. Bard[2], R. Wright[1]; [1]Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, [2]Department of Biology, Indiana University-Purdue University Indianapolis, Indianapolis, IN

To further our understanding of how yeast adapt to low temperatures, we selected 10 psychrophilic yeast strains from ATCC and the Herman Phaff yeast culture collections (Cystofilobasidium capitatum, Cystofilobasidium bisporidii, Cystofilobasidium infirmo-miniatum, Rhodosporidium dacryoideum, Leucosporidium scotti, Sporopachydermia lactativora, Rhodosporidium sphaerocarpum, Cryptococcus vishniacii, Candida psychrophila, and Candida sake). Sterol composition of the mesophilic yeast species, Sacchromyces cereviseae, and the psychrophilic yeast species differ significantly. Interestingly, most of the psychrophilic yeast species contained several sterol metabolites that are not observed in S. cereviseae. We are currently performing mass spectroscopy to identify these intermediates. Amphotericin B and fluconazole, two antifungal drugs that block ergosterol synthesis, were used to further elucidate the role of sterols on growth at low temperatures. Preliminary results show that most of the psychrophilic species have a similar sensitivity to these drugs as S. cereviseae. ER-associated degradation (ERAD) has been shown to modulate overall sterol composition in S. cerevisiae. A taxonomic analysis was conducted to determine whether mechanisms such as ERAD evolved monophyletically or polyphyletically. The sequence of highly conserved regions of the 18S ribosomal DNA and genes involved in ERAD and sterol biogenesis were sequenced in these psychrophilic yeast strains. Preliminary results suggest that the trait of cold tolerance arose multiple times throughout evolutionary history of yeasts.

1604/B56

Endoplasmic Reticulum Proteins Are Required for Cold Adaptation in Saccharomyces Cerevisiae.

J. Loertscher, P. Moore; Chemistry, Seattle University, Seattle, WA

Although the ability of the yeast Saccharomyces cerevisiae to adapt to environmental stresses has been widely studied, relatively little is know about adaptation to cold temperatures. Previous studies by Loertscher and co-workers have shown that proteins required for endoplasmic reticulum-associated degradation (ERAD), such as Ubc7, are required for cold adaptation and may work by affecting membrane sterol composition. Using both solid and liquid growth media, a panel of deletion mutants lacking endoplasmic reticulum-related proteins was tested for sensitivity to cold temperatures. Deletion mutant orm2Δ exhibited notable sensitivity at 10°C when grown on either solid or liquid medium. Interestingly, the deletion mutant of the Orm2 homolog Orm1 was not cold sensitive. Orm2 is an ER-localized protein whose function has been largely uncharacterized. However, a human orm family member has recently been shown by others to be involved in protein maturation of alpha-secretase complex components. We hypothesize that Orm2 may have a role in protein maturation in the endoplasmic reticulum and that this function could be required for cellular adaptation to low temperatures.

Extracellular Matrix and Morphogenesis (1605 – 1622)

1605/B58

A Role for Ephrinb2 in Postnatal Lung Alveolar Development and Elastic Matrix Integrity.

G. A. Wilkinson[1,2], J. C. Schittny[3], D. P. Reinhardt[4], R. Klein[1]; [1]Pediatrics, Medical College of Department, Milwaukee, WI, [2]Molecular Neurobiology, Max-Planck Insititute for Neurobiology, Martinsried, Germany, [3]Insitute of Anatomy, University of Bern, Bern, Switzerland, [4]Faculty of Medicine, McGill University, Montreal, QC, Canada

Alveoli are formed in the lung by the insertion of secondary tissue folds, termed septa, which are subsequently remodeled to form the mature alveolar wall. Secondary septation requires interplay between three cell types: endothelial cells forming capillaries, contractile interstitial myofibroblasts, and epithelial cells. Here we report that postnatal lung alveolization critically requires ephrinB2, a ligand for Eph receptor tyrosine kinases expressed by the microvasculature. Mice homozygous for the hypomorphic knock-in allele ephrinB2ΔV, encoding mutant ephrinB2 with a disrupted C-terminal PDZ interaction motif, show severe postnatal lung defects including an almost complete absence of lung alveoli and abnormal and disorganized elastic matrix. Lung alveolar formation is not sensitive to loss of ephrinB2 cytoplasmic tyrosine phosphorylation sites. Postnatal day 1 mutant lungs show extracellular matrix alterations without differences in proportions of major distal cell populations. We conclude that lung alveolar formation relies on endothelial ephrinB2 function.

1606/B59

Endothelial Pseudo-Vessel Morphogenesis "In Vitro": Requires Transient Changes in Signaling and Gene Expression and Is Differently Supported by Osteoblast and Fibroblast Extracellular Matrix.

P. Manduca[1], D. Visigalli[1], D. Palmieri[1], M. Valli[2]; [1]DiBio, University of Genova, Genova, Italy, [2]Biochemistry, University of Pavia, Genova, Italy

Pseudo-vessel formation in vitro is a reliable test to study the inductive effects of ECM on angiogenesis. We investigated the inductive properties of endogenous ECMs on endothelial cells pseudo-vessels formation "in vitro" and the changes associated, with respect to expression of VEGF-A and its receptors, processing of MMP-2 and activity of putatively relevant signaling pathways. Materials: Osteoblasts are from propagated rat tibiae and Huvec from primary cultures; Eahy962 from the cell Bank at IST, Genova. All fibroblast are characterized and banked at Pavia University. Results: Plating endothelial Eahy926 or Huvec on osteogenic ECM induces the transient activation of MMP-2 and of VEGF-A secretion, immediately preceding pseudo vessel morphogenesis. In Eahy926, VEGF receptor KDR/Flk expression is unchanged and it is transiently co-immunoprecipitated with integrin β1, concomitant with the above changes in MMP-2 and VEGF-A. Also concomitantly and with an equally transient pattern, a slight activation of ERK ½ and a substantial increase in p-38 MAPK activation occur. Control cells on plastic do not show any of the above changes. No relevant changes are detected for FAK and Akt activities. Inhibition of P-p-38 abolishes the formation of pseudo vessels. Collagen (Coll) I deposed by osteoblasts is more extensively glycosylated than that deposed by normal fibroblasts. Inhibition of glycosylation of Coll I during osteogenesis by tunicamycin and enzymatic de-glycosylation of the osteogenic ECM by β-galactosidase inhibit pseudo vessels formation. ECM from normal skin, point and null Coll I mutant fibroblasts (from O.I. patients) or from Coll III mutants (from E.D.) do not support pseudo vessels formation, while fibroblast from an O.I patient with hyper-glycosylated Coll I does. In summary, the induction by ECM of pseudo vessels formation in endothelial cells is mediated in an yet unknow fashion by glycosilated collagen I chains, and dependent from p-38 activation. It is preceded by changes in VGF-A secretion and MMP-2 activity, increased signaling via ERK ½, and association of KDR/Flk with integrin β1. All the above changes are transient and are lost by the time pseudo vessels assume their peculiar morphology.

1607/B60

Mesenchymal Stem Cells and Fibroblasts Stimulate Capillary Morphogenesis by Distinct Proteolytic Mechanisms.

C. M. Ghajar[1], S. Kachgal[1], E. Kniazeva[1], H. Mori[3], S. C. George[1,2], A. Putnam[1,2]; [1]Biomedical Engineering, University of California, Irvine, Irvine, CA, [2]Chemical Engineering and Materials Science, University of California, Irvine, Irvine, CA, [3]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA

During angiogenesis, endothelial cells (ECs) degrade their subjacent extracellular matrix to facilitate invasion. How interactions between ECs and other cells within their microenvironment facilitate this process is unclear. We have utilized a tractable three-dimensional (3D) culture model to determine the proteolytic means by which pre-committed (mesenchymal stem cells, MSCs) or more highly committed (fibroblasts) mesenchymal cells stimulate capillary formation. In the absence of stromal cells, ECs invade the surrounding matrix, but do not form capillaries. However, in the presence of MSCs or fibroblasts, ECs form tubular structures, demonstrate apical-basal polarity, and induce pericyte-like behavior by both mesenchymal cell types, all within 7 days. Further, in response to MSCs or fibroblasts, ECs upregulate their expression of several extracellular protease transcripts by day 3, implying that the upregulation of at least a subset of these proteases is necessary for capillary formation to occur. The administration of broad spectrum protease inhibitors, more specific function blocking antibodies, and highly specific shRNA confirmed this notion while revealing a distinct trend: MSC-stimulated capillary formation relied solely on matrix metalloproteinases (MMPs, specifically MMP14), while both MMP14 and a member of the plasminogen activator axis (urokinase-type plasminogen activator receptor, uPAR) mediated fibroblast-stimulated capillary morphogenesis. While other studies have established roles for MMP14 and uPAR in monotypic culture, the differential dependence on these proteolytic systems in the heterotypic cultures used here demonstrate the need for more complex culture systems to model angiogenesis.

1608/B61

Barx2 Is an Important Regulator of Extracellular Matrix Remodeling and Ocular Glands Development.

C. Tsau[1], R. Meech[2], H. Makarenkova[1,2]; [1]Neurosciences Institute, San Diego, CA, [2]Department of Neurobiology, The Scripps Research Institute, La Jolla, CA

We have investigated the role of the homeodomain transcription factor, Barx2 in branching morphogenesis of the mouse ocular glands: lacrimal gland (LG) and Harderian gland (HG). Barx2 -/- mice also showed dramatic defects in both ocular glands and eyelid development. Thus, LGs were either absent or poorly developed in Barx2-/- mice. In addition, Barx2-/- lacrimal glands showed a strong reduction of MMP2, MMP3, and MMP9 mRNA levels relative to wildtype glands. This finding is supported by the results of a Barx2 gain of function experiment in the A253 epithelial cell line where ectopic Barx2 expression promoted migration of cells through the matrix and upregulated the expression of MMPs. Barx2 -/- mice also showed dramatic defects in Harderian gland and eyelid development. The Harderian gland was completely absent in 99% of studied Barx2 -/- mouse embryos, while 85% of embryos showed defects in eyelid fusion. We also examined the gene expression profiles of Barx2+/- and Barx2-/- LGs using the Mouse Extracellular Matrix and Adhesion Molecules RT-Profiler™ PCR. The array results show decrease of expression level of several cell matrix and adhesion molecules, which is consistent with other observations. Together these data suggest that Barx2 plays an essential role in cell-cell and cell-matrix interactions regulates branching morphegenesis of the LG, HG and eyelid development.

1609/B62

Change in Cell Shape Is Required for Matrix Metalloproteinase-Induced Epithelial-Mesenchymal Transition of Mammary Epithelial Cells.

C. Nelson[1,2], D. Khauv[3], M. Bissell[1], D. Radisky[3]; [1]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, [2]Chemical Engineering, Princeton University, Princeton, NJ, [3]Cancer Biology, Mayo Clinic Cancer Center, Jacksonville, FL

Cell morphology dictates response to a wide variety of stimuli, controlling cell metabolism, differentiation, proliferation, and death. Epithelial-mesenchymal transition (EMT) is a developmental process in which epithelial cells acquire migratory characteristics, and in the process convert from a cuboidal epithelial structure into an elongated mesenchymal shape. We had shown previously that matrix metalloproteinase-3 (MMP3) can stimulate EMT of cultured mouse mammary epithelial cells through a process that involves increased

expression of Rac1b, a protein that stimulates alterations in cytoskeletal structure. We show here that cells treated with MMP-3 or induced to express Rac1b spread to cover a larger surface, and that this induction of cell spreading is a requirement of MMP-3/Rac1b-induced EMT. We find that limiting cell spreading, either by increasing cell density or by culturing cells on precisely defined micropatterned substrata, blocks expression of characteristic markers of EMT in cells treated with MMP-3. These effects are not caused by general disruptions in cell signaling pathways, as TGF--induced EMT is not affected by similar limitations on cell spreading. Our data reveal a previously unanticipated cell shape-dependent mechanism that controls this key phenotypic alteration and provide insight into the distinct mechanisms activated by different EMT-inducing agents.

1610/B63

Raf-Induced Matrix Metalloproteinase-9 (MMP-9): a Disorganization Node in a Breast Cancer Progression Model.

A. Beliveau[1], J. Mott[1], A. Lo[1], P. A. Kenny[2], P. Yaswen[1], J. L. Muschler[3], M. Bissell[1]; [1]Life Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, [2]Albert Einstein Cancer Center, Albert Einstein College of Medicine, Bronx, NY, [3]California Pacific Medical Center Research Institute, San Francisco, CA

The ability of breast epithelial cells to organize into polarized three-dimensional (3D) structures correlates with their normal or malignant status. Using a versatile model of acinar morphogenesis, where cells are embedded in a laminin-rich gel, we previously demonstrated that individually inhibiting key signaling pathways in human breast cancer cells one by one leads to phenotypic 'reversion' of the malignant 3D structures. Here we explore the positive or negative regulatory 'signaling nodes' that are shared by all the reverting agents examined during the morphogenesis of phenotypically normal structures. Examining gene expression arrays before and after reversion, we observed the influence of reduced Raf/MEK/ERK signaling on tissue polarity and growth arrest. To examine this more closely, we evaluated increased signaling by introducing a Raf-estrogen receptor fusion protein into breast cells, where the presence of estrogen induced Raf activity. Decreased signaling was evaluated using small molecule inhibitors or depletion of MEK1/2 with shRNA. Decreased signaling resulted in formation of growth arrested polarized structures whereas increased signaling exerted a potent disruption of tissue organization and reinitiated cellular proliferation in growth arrested polar structures. These experiments implicated MMP-9 as a key molecule in this process. Using chemical inhibitors or shRNA against MMP-9, led to phenotypic reversion similar to decreased Raf/MEK/ERK signaling. Conversely, elevated MMP-9 activity, achieved by introduction of activatable MMP-9 into non-malignant cells, resulted in disrupted polarity and reinitiated cellular proliferation similar to increased Raf/MEK/ERK signaling. These data suggest that induction of Raf or MMP-9 in non-malignant polarized epithelia is sufficient to disrupt tissue architecture and reinitiate proliferation. That inhibition of Raf or MMP-9 restored the ability of malignant breast epithelial cells to form quiescent polarized structures implicates Raf/MEK/ERK and MMP-9 as a 'disorganization node' for destroying tissue polarity and inducing growth, shared by a number of important regulatory pathways in epithelial cells.

1611/B64

The Intersection of Planar Cell Polarity and ECM Remodeling during Collective Migration and Invasion.

J. R. Jessen[1,2], A. Latimer[1,2], A. Cantrell[1]; [1]Medicine, Vanderbilt University Medical Center, Nashville, TN, [2]Cancer Biology, Vanderbilt University Medical Center, Nashville, TN

During zebrafish gastrulation lateral mesodermal cells undergo a transition from individual to collective modes of directed migration towards the dorsal body axis. This transition is dependent upon the Wnt/Planar Cell Polarity (PCP) pathway shown to regulate cell polarity within the plane of a tissue during embryonic development. In *trilobite* (*vangl2*) mutant embryos, mesodermal cells fail to become polarized and collective movement is significantly disrupted. However, while cell polarity is mechanistically linked to efficient directed migration, the loss of polarity does not completely abolish motility. This suggests the involvement of additional proteins that act in coordination with Wnt signaling to regulate distinct cell properties. Our objective was to determine whether changes in pericellular proteolysis and ECM degradation contribute to planar cell polarity. Using a combination of molecular methods and time-lapse imaging techniques, our results show that matrix metalloproteinase 14 (Mmp14) is required for proper cell polarity and orientation of membrane processes. Mmp14 is a membrane-anchored protease capable of degrading many ECM components and is considered the principal protease controlling tumor cell invasion. We have identified a genetic interaction between *mmp14* and several Wnt/PCP genes including *trilobite* (*vangl2*) and *knypek* (*glypican4*). Our results show a striking correlation between the onset of collective movement and (1) the requirement for Mmp14 and Vangl2 function and (2) the assembly of a polarized fibronectin network adjacent to mesodermal cells. Similar to data from frog and mouse, we have now linked Vangl2 function with proper fibronectin distribution during zebrafish gastrulation. Intriguingly, our results demonstrate that human VANGL2 also influences fibronectin in invasive cancer cells. Our cell culture data support the notion that VANGL2 regulates the localization of MMP14 to specific cellular domains. We conclude that the intersection of pathways regulating pericellular proteolysis and cell polarity may represent a conserved mechanism whereby cell populations coordinate the formation of polarized membrane processes necessary for collective migration and perhaps invasion.

1612/B65

Rho Kinase (Rock) Signaling Regulates Cleft Elongation during Mouse Submandibular Salivary Gland Branching Morphogenesis.

W. P. Daley, K. Gulfo, M. Larsen; Biological Sciences, University at Albany, State University of New York, Albany, NY

Cleft initiation and elongation are the two initial steps in submandibular salivary gland (SMG) branching morphogenesis, and may result from the localized contraction of the actin cytoskeleton and basement membrane remodeling at sites of cleft formation. A major pathway promoting cytoskeletal contraction involves signaling through Rho GTPase and its downstream effector, Rho kinase (ROCK). We investigated the effects of ROCK inhibition and decreased cytoskeletal contractility on cleft formation and tested the hypothesis that ROCK signaling affects basement membrane remodeling during SMG branching morphogenesis. Mouse E13 SMG organ explants were cultured ex vivo for 24 hours in the presence of the ROCK inhibitors, Y27632 and H-1152, the myosin ATPase inhibitor, blebbistatin, or vehicle control. Morphometric analysis revealed that ROCK and myosin inhibition resulted in a decreased bud count and in an increase in the number of initiated, but not elongated, clefts. ROCK-inhibited SMGs also exhibited decreased phosphorylation of the ROCK effector, the regulatory

light chain of non-muscle myosin II, and an accompanying decrease in overall gland thickness, both of which are indicative of decreased cytoskeletal tension. Since previous studies indicated that the extracellular matrix protein fibronectin (FN) is required for SMG epithelial branching morphogenesis and that ROCK signaling can regulate FN assembly, we examined FN fibrils in treated SMGs. Confocal microscopy revealed FN disorganization in the basement membranes of both ROCK- and myosin-inhibited SMGs, as well as increased FN localization within epithelial cells. Furthermore, studies with SCA-9 cells, a salivary gland epithelial cell line, also revealed a decrease in the amount of deoxycholate-insoluble FN fibrils in the matrices of cells cultured with ROCK and myosin inhibitors, further suggesting a defect in FN matrix secretion and/or assembly when cytoskeletal contractility is inhibited. We conclude that signaling through ROCK, leading to increased cytoskeletal tension, is an important determinant of cleft elongation, which may function through altering FN remodeling in developing SMGs.

1613/B66

Tissue Microenvironmental Regulation of Normal and Neoplastic Mammary Epithelial Morphogenesis.

A. Ewald[1], A. Brenot[2], Z. Werb[2]; [1]Cell Biology & Center for Cell Dynamics, Johns Hopkins Medical School, Baltimore, MD, [2]Anatomy, University of California, San Francisco, CA

Epithelial organs are built and remodeled through the movement of groups of interconnected cells. We have shown that cells in elongating ducts migrate collectively, rearrange dynamically and lack leading cellular extensions. We used 3D confocal timelapse imaging to visualize the coordinate motility of both luminal and myoepithelial cells during normal branching morphogenesis and in response to interference with Rac, myosin light chain kinase (MLCK), and Rho kinase (ROCK) signaling. New duct initiation from these preinvasive structures requires Rac and MLCK. Transition back to a quiescent bilayer requires ROCK. In each molecular perturbation, the location and kinetics of the myoepithelial cells predicts the invasive outcome. We visualized the cell adhesional basis of branching morphogenesis using combined electron and light microscopy approaches and have identified dramatic coordinate changes in tight, adherens and desmosomal junctions during morphogenesis. These junctional remodeling events depend on small GTPase activity, with ROCK promoting junctional assembly and Rac junctional disassembly. We have found that collective epithelial migration is regulated by extracellular matrix proteins and matrix metalloproteinases (MMP) and have found that the cellular mechanisms of invasion are dependent on the 3D extracellular matrix (ECM) environment, with distinct growth factor and MMP requirements in different matrices. The cellular mechanism of invasion is dependent on the local properties of the tissue microenvironment, such that single ducts can invade by different mechanisms if presented with different ECM choices. Finally, we are contrasting the cellular mechanisms of epithelial morphogenesis in: normal mouse mammary epithelium, epithelium from mouse models of breast cancer and primary human mammary epithelium. Based on 4D timelapse movies, common cellular mechanisms underlie normal and neoplastic mammary epithelial morphogenesis in mice and in humans.

1614/B67

HoxA9 Regulates Stromal-Mammary Epithelial Interactions through Modulation of BRCA1 Expression.

J. Mouw[3,4], P. Gilbert[1,2], M. Unger[5], J. Lakins[1,3,4], M. Gbegnon[1], M. Nuth[1], V. Clemmer[6], T. Colligan[5], M. Benezra[7], J. Licht[7], M. Feldman[1], N. Boudreau[3], B. Weber[5,8], V. Weaver[1,3,4]; [1]IME/Pathology and Lab Medicine, UPENN, Philadelphia, PA, [2]Current: Department of Microbiology and Immunology/Baxter Laboratories, Stanford University, Stanford, CA, [3]Department of Surgery and Anatomy, UCSF, San Francisco, CA, [4]Center for Bioengineering and Tissue Regeneration, UCSF, San Francisco, CA, [5]Abramson Family Cancer Research Institute, UPENN, Philadelphia, PA, [6]St. Francis Hospital, Wilmington, DE, [7]Mount Sinai School of Medicine, New York, NY, [8]Current: GlaxoSmithKline, Philadelphia, PA

Stromal-epithelial interactions drive development and maintain tissue homeostasis through a network of soluble and insoluble factors that operate within a three-dimensional (3D) tissue. Genetic and epigenetic changes in mammary epithelial cells (MECs) cooperate with a modified tissue microenvironment to drive malignant transformation of the breast. Hox genes play a critical role in tissue development, and tumors often express altered levels of homeobox genes although the significance of this observation to tumor progression is unclear. Using global expression analysis of matched tumor/normal human breast tissues we found that expression of the homeobox gene HoxA9 was significantly lower in tumors. Q-RT-PCR, in situ, and immunohistochemical analysis revealed that HoxA9 was primarily expressed in the breast epithelium and significantly reduced in a cohort of primary breast tumors and aggressive breast cancer cell lines. Normalizing HoxA9 levels increased BRCA1 expression in breast tumor cells and repressed their growth and survival and reverted their malignant behavior in a three dimensional basement membrane assay and in vivo. Knocking down HoxA9 using shRNA reduced BRCA1 levels and enhanced the growth and survival and disrupted the acinar morphogenesis of nonmalignant mammary epithelial cells. HoxA9 consensus binding sequences were identified in the BRCA1 promoter and confirmed by ChIP and luciferase analysis and validated by mutational studies. Expression of a wild-type BRCA1 or a BRCA1 mutant phenocopied the HoxA9-dependent inhibition of the breast cancer cells, and shRNA knockdown of BRCA1 or co-expression of the BRCA1 mutant promoted growth and disrupted acinar morphogenesis of nonmalignant mammary epithelial cells. Because compromising BRCA1 function prevented HoxA9 from reverting the malignant behavior of breast tumor cells in culture and in vivo, we suggest that HoxA9 could regulate breast tumor progression by normalizing stromal-epithelial interactions through modulation of BRCA1 expression. Our results offer a plausable explanation for why sporadic breast cancers often have decreased BRCA1 expression even in the absence of genetic deletions, methylation or haplo insufficiency.

1615/B68

Characterization of the Cellular Behaviors Underlying the Asymmetric Migration of the Lateral Plate Mesoderm during Gut Looping in Zebrafish.

C. Yin[1], K. Kikuchi[2], K. Poss[2], D. Stainier[1]; [1]Department of Biochemistry, University of California, San Francisco, San Francisco, CA, [2]Cell Biology, Duke University Medical Center, Durham, NC

While most vertebrate internal organs adopt asymmetric positions with respect to the midline, little is known about the cellular changes and tissue movements that occur downstream of left-right gene expression to produce this asymmetry. In the vertebrate digestive system, the liver and pancreas are located on the left and right side of the midline, respectively. In zebrafish, the asymmetric positioning of these two organs is

the consequence of the leftward looping of the gut tube. Failure of gut looping often leads to the 'duplication' of the digestive organs across the midline. Previously we have shown that the asymmetric migration of the left and right lateral plate mesoderm (LPM) displaces the gut to the left. To further analyze the cellular behaviors underlying this asymmetric migration of the LPM, we utilized *Tg(dhand:egfp)* transgenic zebrafish that expresses GFP in the LPM. We found that the asymmetric migration of the LPM is not due to different rates of cell proliferation or apoptosis in the left and right LPM. Instead, our confocal imaging analyses revealed that the migration of the LPM consists of multiple steps and that the cells in the left and right LPM exhibit distinct migration behaviors. Furthermore, we detected a dynamic deposition of the extracellular matrix (ECM) between the LPM and the gut tube that is closely related to the migration behaviors of the LPM cells. Based on mutant analyses, we found that loss of epithelial polarity and *dhand* function both lead to failure of gut looping. In these mutant embryos, the ECM deposition is altered and the migration behaviors of the left and right LPM cells become symmetrical. These results suggest that dynamic cell-matrix interaction and differential cell migration behaviors may serve as the driving force for the asymmetric LPM migration and the consequent leftward looping of the gut.

1616/B69

Cleftin: a Novel Fibronectin-Induced Gene that Promotes Branching Morphogenesis.

T. Onodera[1], T. Sakai[2], K. Yamada[1]; [1]NIDCR, NIH, Bethesda, MD, [2]Graduate School of Dentistry, Osaka University, Suita, Japan

Branching morphogenesis is a fundamental process shared by many developing organs. In this process, the epithelium of the mouse salivary gland forms a cleft that deepens to generate lobules. Cleft formation involves the conversion of cell-cell to cell-matrix adhesions. Fibronectin is known to appear transiently and focally in forming cleft regions, accompanied by an adjacent loss of E-cadherin localization, but the mechanism is still unknown. We hypothesized that cleft epithelial cells express new functional genes that regulate cleft formation. We searched for novel genes that are expressed in cleft but not bud epithelium using laser microdissection and T7-SAGE libraries. A BTB/POZ domain-containing protein that we termed "cleftin" showed differential expression in the cleft epithelium. Knockdown of cleftin using siRNAs inhibited branching of both developing mouse salivary glands and lungs, indicating an important role for cleftin in branching morphogenesis. Expression of cleftin in developing salivary submandibular glands was maximal at embryonic day 13, a period of extensive gland branching. In situ hybridization revealed highly localized expression of cleftin mRNA around the tip (base) of the cleft. To determine whether cleftin can be induced, we tested several matrix molecules. Fibronectin rapidly induced cleftin expression. Functional analysis of branching morphogenesis using stably transfected Madin-Darby canine kidney (MDCK) cells showed that increased cleftin expression decreased E-cadherin and promoted branching in 3D collagen gels. These phenotypes of cleftin-expressing cells resemble those seen in epithelial-mesenchymal transition (EMT), so we speculated that cleftin expression might induce focal, partial EMT to regulate cleft propagation. We found that fibronectin rapidly induced not only cleftin but also the EMT transcription factor slug (snail2). Cleftin expression itself induced slug, and siRNA knockdown of slug inhibited branching. Our findings present a novel mechanism regulating organ branching in which we propose that local matrix protein accumulation induces focal expression of cleftin, which in turn induces slug to promote focal EMT and cleft propagation.

1617/B70

Contact-Inhibition of Motility and Its Role in Multicellular Aggregate Formation.

M. Pope, A. Asthagiri; California Institute of Technology, Pasadena, CA

Epithelial tissues are dynamic structures that provide a polarized physical barrier between the external and internal environments. Continuous remodeling of epithelial structures plays an important role in development and regeneration, but also contributes to caner development. Thus the clinical and physiological significance of epithelial structures is well established; however the molecular mechanisms that mold, maintain and disrupt them are not well understood. This work examines the regulation of epithelial morphogenesis by characteristics of the cellular microenvironment. We have established culture conditions that induce a non-transformed mammary epithelial cell line to form two-dimensional cell clusters of varying sizes and have developed quantitative metrics to parse the contributions of individual molecular signals to aggregate formation. We find that aggregate size exhibits a biphasic dependence on surface adhesivity. This finding is in contrast to published paradigms and underscores the role of cell motility in cluster formation. In fact, quantification of single cell migration speeds reveals that surfaces that promote greater cell motility also facilitate the formation of larger cell clusters. We also find culture conditions that promote aggregation to decrease the elasticity of cell-cell collisions. Taken together, these results suggest contact-inhibition of cell motility to play a governing role in the assembly of multicellular aggregates. Using molecular tools that modulate cadherin and integrin function, we are currently examining the interplay of cell-matrix and cell-cell interactions in the context cell adhesion decisions. In summary, this work provides new insights into the regulation of epithelial morphogenesis and will help to advance our understanding of its role in health and disease. Furthermore, our results provide design principles that may be useful for engineering therapeutics capable of reshaping epithelium.

1618/B71

Low Substratum Stiffness Downregulates B1 Integrin Expression and Prevents TGF-β1-induced Epithelial-Mesenchymal Transition in Proximal Tubular Epithelial Cells.

W. Chen, H. Lin, M. Tang; Department of Physiology, National Cheng Kung University, Tainan, Taiwan

Recent studies indicate that substratum stiffness regulates cell behavior, i.e. stem cell fate, cancer cell phenotype, apoptosis and growth factor-induced cell differentiation. However, whether substratum stiffness modulates epithelial-mesenchymal transition (EMT) induced by transforming growth factor β1 (TGF-β1) has not been studied. To elucidate the role of substratum stiffness in TGF-β1-induced EMT, we employed type I collagen-coated polyacrylamide gel of various stiffness. We observed that TGF-β1-induced EMT in LLC-PK1 cells and primary culture of mice proximal tubular cells (mPT) were manifested by upregulation of α-SMA, fibronectin, β1 integrin and downregulation of E-cadherin. TGF-β1-induced EMT changes could be abrogated by low substratum stiffness in both cell line and strain. Interestingly, unlike the phenotype of primary mPT cells cultured on stiffer substratum, primary mPT cells cultured under stiffness of lower than 2415 Pa did not spread out but maintained tubular structure throughout 7 days regardless of the presence of TGF-β1. To further elucidate the mechanism, we found that TGF-β1-induced EMT could be blocked by neutralizing β1 integrin antibody. On the other hand, low

substratum stiffness downregulated β1 integrin protein and mRNA level regardless of the presence of TGF-β1 via transcriptional and post-transcriptional regulation. Finally, low substratum stiffness reduced phosphorylation of Smad3, ERK and expression of ERK regardless of the presence of TGF-β1. Taken these data together, low substratum stiffness inhibits the expression of β1 integrin, which might mediate TGF-β1-induced EMT. On the other hand, substratum stiffness alone modulates different signal pathways which were related to TGF-β1 signal. This study facilitates our understanding of how substratum stiffness regulates the normal physiological function and the pathological development of renal fibrosis.

1619/B72

Gene Expression Profiling of Sphingosine-1-Phosphate-Induced Endothelial Cell Invasion.

S. Su, H. Kwak, E. Mendoza, K. Bayless; Molecular & Cellular Medicine, Texas A&M University System Health Science Center, College of Medicine, College Station, TX

Angiogenesis is a multi-step process consisting of the activation of the endothelial cells (ECs), degradation of the basement membrane, invasion and proliferation of ECs, lumen formation, and stabilization. Such complexity reveals that the orchestration of individual genes and multiple signaling pathways is required for controlling the process of sprouting angiogenesis. To better understand the mechanisms that direct the transformation of adherent ECs on the surface of a collagen matrix to multicellular invading sprouts, we have performed an analysis of differential gene expression using a defined in vitro model of EC invasion driven by the combination of sphingosine-1 phosphate (S1P), basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF). We have found that a series of genes that regulate endothelial cell-cell junctions and extracellular matrix (ECM) interactions are systematically regulated during EC invasion. Among them, a cohort of cell adhesion molecule (CAM) genes involved in adherens junction and cell-matrix interaction are upregulated in our microarray analysis, whereas a set of genes associated with tight junction (TJ) formation are downregulated. In addition, numerous genes encoding ECM proteins and proteases are induced, indicating that biosynthesis and remodeling of ECM is indispensable for sprouting angiogenesis. A Disintegrin and Metalloproteinase with Thrombospondin Repeat-1 (ADAMTS1) expression increased markedly over time. Silencing of ADAMTS1 expression in ECs resulted in decreased invasion density and distance, and enlarged lumen diameter, markedly altering invasion responses. Furthermore, we demonstrated differential expression of multiple members of the Wnt and Notch pathways as ECs undergo invasion. Functional experiments demonstrate that Notch inhibition by gamma-secretase inhibitor and activation by overexpression of Delta-like 4 (Dll4) and Notch intracellular domain (NICD) stimulated and inhibited EC invasion responses, respectively. Collectively, these data indicate that this system closely recapitulates in vivo angiogenic events and highlight the utility of employing such a system to study EC morphogenesis in three dimensions using defined conditions.

1620/B73

Temporospatial Expression of Lysyl Oxidase in the Presumptive Dermis of Chick Limb Bud.

Y. Yamazaki, Y. Mikami, Y. Namba, K. Isokawa; Anatomy, Nihon University School of Dentistry, Tokyo, Japan

Lysyl oxidase (LOX) activity is involved in the formation of crosslinking between and within the molecular units of elastin and of collagen. The enzyme converts their lysine side chains into allysines, which stabilize macromolecular assembly of extracellular fibers. We showed temporospatial progress of elastic system fiber formation and the coordinated accumulation of collagen fibers in the dermis of the developing chick leg bud (Yamazaki et al., 2007). Because this developmental process must be closely related to LOX activity in the dermis, we have investigated its temporospatial expression by immunohistochemistry and real-time quantitative RT-PCR; laser capture microdissection system was used for isolating tissue in specific regions of the dermis precisely. The results of PCR analysis showed that the expression of LOX mRNA is upregurated at ED13 and increased dramatically at ED17. Intensity of immunohistochemical staining for LOX in the dermis was coordinately increased at ED17. As noted previously, microfibrillar fibers (MFs) visualized with an antibody to chick fibrillin-2 have been seen in the dermis by embryonic day 4 (ED4), while elastin deposition detected first in a punctate manner at ED13 became linear along subectodermal vertical MFs by ED17. Collagen fibers accumulated in the dermis as late as ED10. These findings suggest that temporal pattern of LOX expression after the onset of elastin deposition is correlated not only with collagens but also elastic fiber formation in the dermis of developing chick limb bud.

1621/B74

A Key Role for Dystroglycan in Mammary Epithelial Morphogenesis and Function, Established In Vivo.

J. E. Fata[3], R. Mohajer[1], M. Singh[1], D. Leonoudakis[1], K. P. Campbell[2], J. Muschler[1]; [1]California Pacific Medical Center Research Institute, San Francisco, CA, [2]University of Iowa and HHMI, Iowa City, IA, [3]City University of New York, Staten Island, NY

The basement membrane receptor dystroglycan has been implicated in epithelial cell functions from laminin assembly to signaling for epithelial polarization, cell survival, and tissue-specific gene expression. Moreover, loss of dystroglycan function is widely observed in carcinoma cells and is likely to contribute to disease progression. However, an in vivo assessment of dystroglycan's role in epithelial development and function in adult tissues has not yet been reported. We have employed the conditional knockout of dystroglycan expression in vivo, by Cre-Lox recombination, to assess dystroglycan function in the epithelial lineage of the mammary gland. The floxed-dystroglycan transgenic mouse line was crossed with mice expressing Cre transgene driven either by the keratin-14 promoter (K14-Cre) or by the mouse mammary tumor virus promoter (MMTV-Cre). The K14-Cre-mediated knockout occurs very early in mammary gland development, whereas the MMTV-Cre mediated knockout occurs several weeks after birth. Deletion of dystroglycan by K14-Cre expression produced clear defects in mammary gland development and function. At 8 weeks of age, the gland of female dystroglycan knockout mice displayed a marked reduction in outgrowth. At mid-pregnancy (d14.5), defects of outgrowth were no longer evident, but defects were evident at the stage of lactation, with impaired alveolar development and more than half of the mothers (n=15) failing to nourish their pups. The MMTV-Cre mediated knockout of dystroglycan displayed a more subtle phenotype from that mediated by K14-Cre, reflecting differences in the timing and/or population specificity of the targeted knockout mediated by the two Cre-expressing lines. Results presented here demonstrate clear defects in tissue development and function resulting from the loss of dystroglycan within an epithelial cell lineage of adult transgenic mice.

1622/B75

XGIPC/Kermit2 Regulates Integrin Adhesion during Xenopus Gastrulation.

E. Spicer, M. Marsden; Biology, University of Waterloo, Waterloo, ON, Canada

In Xenopus, a Fibronectin (FN) matrix is necessary for morphogenetic movements occurring at gastrulation and defines domains of cellular rearrangements. As both FN and integrin are expressed in an unrestricted manner in the early embryo, the spatial and temporal regulation of FN matrix assembly must occur at a higher level than simply protein expression. In vitro, a change in integrin/FN interactions can be shown to occur at the initiation of gastrulation suggesting that changes in integrin binding are responsible for this behaviour. We have demonstrated that this alteration in integrin function can be partially attributed to interaction between the integrin α5 subunit and XGIPC/kermit2. Yeast two hybrid assays reveal a strong interaction between kermit2 and the cytoplasmic domain of both integrin α5 and α6. Kermit2 can be co-immunoprecipitated with integrin α5β1 in vivo. Using site directed mutagenesis these interactions can be mapped to the central PDZ domain of kermit2. Furthermore, over-expression of a dominant negative kermit2 (DNkermit2) inhibits gastrulation in vivo. This effect is upstream of matrix assembly as expression of DNkermit2 results in the inhibition of FN matrix assembly on the blastocoel roof. Blastocoel roof cells expressing a DNkermit2 retain the ability to adhere to FN substrates in vitro. However, unlike cells derived from control embryos they cannot be induced with activin to migrate. We have observed that cells expressing DNkermit2 have difficulty in maintaining persistent protrusions suggesting a lack of substrate adhesion. Antibody internalization assays indicate that a DNkermit2 inhibits integrin α5β1 recycling in Xenopus A6 cells. Together these data indicate that kermit2 plays a role in integrin recycling, and that recycling is essential for changes in integrin mediated cell behaviours. We propose that kermit2 plays a central role in the spatial and temporal regulation of integrin adhesion observed during early Xenopus gastrulation.

Cell Attachment to the Extracellular Matrix (1623 – 1633)

1623/B76

The Study of the Correlation between Focal Adhesion Kinase Phosphorylation and Ability of Cell Adhesion.

J. Su[1], C. Chen[2], C. Ay[1]; [1]Department of Biomechatronic Engineering, National Chiayi University, Chiayi City, Taiwan, [2]Department of Biochemical Science and Technology, National Chiayi University, Chiayi City, Taiwan

This study investigated the relationship between the cell adhesion and activation of focal adhesion kinase (FAK) when the human umbilical vein endothelial cells cultured in a coating of collagen protein and fibronectin of biomedical materials-dimethyl siloxane (Polydimethylsiloxane, PDMS). The electrophoresis force is used to measure the attached ability of HUVECs then driving voltage of electrodes to produce the electrophoresis force meant the adhesive ability of cell. The difference between the adhesive ability of cell and activation of FAK is compared under HUVECs cultured in a coating of collagen protein and fibronectin on PDMS. Then find out which extracellular matrix (ECM) cells can provide a more stable growth environment for cell for the collagen protein and fibronectin. The results revealed that when cells cultured in a collagen protein coating on the PDMS which adhesive ability is more instability. The adhesive ability for the second and fifth hours increased and the activation of the FAK also increased for the second and fifth hours. When cells cultured in a coating of the fibronectin attached PDMS which adhesive ability is more stable. It showed that adhesive ability increased as cultured time and FAK activation phenomenon also increased as time. On the activation of FAK, the experiment showed the significant relationship between the adhesive ability of cell and FAK activation phenomenon. This study attempts to join three inhibitors, AG18, Genistein and LY294002 to inhibit protein phosphorylation. It shows Genistein on the adhesive ability and the inhibition of protein activation is more effective. However FAK may be an important role on the adhesive ability of cell.

1624/B77

Vinculin Recruits G-Actin to Form an Actin Nucleus from Which Actin Filaments Arise.

K. Wen, P. Rubenstein, K. DeMali; Department of Biochemistry, University of Iowa, Iowa City, IA

Cell adhesion, migration, proliferation and survival require a functional linkage of the extracellular matrix with the actin cytoskeleton. This linkage is brought about by adhesion complexes composed of integrin receptors and associated proteins. Accumulating evidence suggests that adhesion complexes promote actin assembly from their cytosolic face in order to establish a mechanical linkage with the extracellular matrix. Previous work implicates the vinculin tail (Vt 881-1066) in this process by virtue of its ability to bundle actin filaments. However, the nature and the consequence of this interaction is not well understood. In this study we examined the consequence of vinculin binding to actin using yeast actins (proteins 90% homologous to mammalian β- and γ- non-muscle actin) to examine the effect of VT binding on actin filament structure and dynamics. Vt binds to G-actin in low salt solution (non-polymerizing) and by itself causes the G-actin to polymerize without the addition of added salt. EM analysis of these filaments demonstrated that Vt and G-actin form long single filaments at substoichiometric Vt concentrations and massive ordered actin filament bundles at stoichiometric Vt concentrations. To determine whether these filaments reflected a normal F-actin structure, we assessed the behavior of Vt with a mutant yeast actin, S265C, labeled with pyrene both here and on the C-terminus. In the F-form, these two probes from different strands interact to form a pyrene excimer. Such an excimer formed when G-actin and Vt were mixed in the absence of polymerizing salts. Vt also accelerated the formation of F-actin under normal polymerizing conditions and also seems to affect F-actin structure based on fluorescence quenching data. These results demonstrate that vinculin recruits G-actin to form an actin nucleus from which actin filaments arise and accelerates the rate of actin polymerization. Furthermore, vinculin alone can confer the ability to link nascent adhesion receptors to the underlying actin cytoskeleton.

1625/B78

Patterned Nanometer-Scale Fibers of Fibronectin for Studying Cellular Responses to Anisotropic Microenvironments.

J. L. Werbin[1], W. F. Heinz[1], D. Pesen[3], P. Soucy[4], L. Romer[5,4], D. B. Haviland[2], J. H. Hoh[1]; [1]Physiology, Johns Hopkins School of Medicine, Baltimore, MD, [2]Applied Physics, Royal Institute of Technology, Stockholm, Sweden, [3]Anatomy & Structural Biology, Albert Einstein

College of Medicine, New York, NY, [4]Biomedical Engineering, Johns Hopkins School of Medicine, Baltimore, MD, [5]Anesthesiology and Critical Care Medicine, Johns Hopkins School of Medicine, Baltimore, MD

Micropatterning offers a powerful approach to understanding how cells respond to spatial and biochemical cues in the microenvironment. In this context, one of the most fundamental organizational units is the fiber, and many components of the extracellular matrix - such as fibronectin and collagen - have a fibrous structure. In some biological settings fibers in the ECM appear to be isotropic, while in others fibers are highly oriented. This suggests that fiber orientation may play an important functional role. To study the functional effects of fiber anisotropy we have developed an approach based on electron beam lithography, to produce fibers of fibronectin that have a thickness on the order of 100 nm and lengths of several hundred micrometers or more. The density, orientation and persistence length of these fibers can be experimentally controlled, offering the opportunity to probe cell responses as a function of these parameters. This system also permits the anisotropy to be carefully quantified using a newly developed information theoretic approach. Initial characterization demonstrates that these fiber patterns support cell attachment and spreading, and that cells recognize and respond to the anistropic distribution of the protein.

1626/B79

Long-Term Viability, Contractility, and Cytostructural Assembly of Cardiomyocytes and MSCs in 3D-Elastic Gels.

A. L. Zajac[1], F. Rehfeldt[1,2], C. Carag[1], D. Discher[1]; [1]Molecular & Cell Biophysics Lab, University of Pennsylvania, Philadelphia, PA, [2]Department of Physics, University of Goettingen, Goettingen, Germany

Injection of stem cells into cardiac tissue has met with mostly negative results over the last number of years, with many cells dying and few signs of differentiation. Towards developing culture systems to explore the underlying cell and molecular issues, we have encapsulated both embryo-derived cardiomyocytes (CMs) and mesenchymal stem cells (MSCs) in 3D elastic hydrogels of hyaluronic acid (HA) containing collagen-I or gelatin for cell adhesion. Previous work in our lab has demonstrated the importance of matrix elasticity to muscle differentiation in culture. By varying the concentration of HA, we can tune the hydrogel stiffness, allowing us to match the elasticity of cardiac muscle, or even the fibrotic stiffness of a cardiac infarct, while at the same time conserving the dimensionality of cell-matrix interactions from in vivo. Both cell types remain viable for one week or longer after encapsulation in HA hydrogels based on a metabolic reduction assay. Adhesion within the 3D matrix on the dorsal and ventral surface of the cells was confirmed by confocal microscopy of vinculin immunostaining in MSCs. CMs also maintained their rhythmic contractions within 3D, confirming cell attachment to the surrounding hydrogel through movement of fiduciary marks around the cells during beating. Although MSCs and CMs encapsulated within 3D polymerized gels remained round during the course of experiments, they had different cytoskeletal responses. MSCs, by 24hrs, began to send out actin-rich projections in all directions, and these grew in size and number for about 5 weeks. The surface of CMs remained smooth over one week in culture, but the cells contained many sarcomeric alpha-actinin-positive striated myofibrils oriented parallel to the round surface of the cell. Elasticity has an unknown role as of yet. We have thus begun to characterize a hydrogel culture method that maintains the characteristic striation and rhythmic beating of differentiated cardiomyocytes in vitro, allowing comparison of MSC and CM responses to the mechanical microenvironment of both the compliant normal heart and the rigid infarcted heart.

1627/B80

Control of Cell Proliferation and Adhesion Dynamics by Microscale Topographical Cues.

A. A. Patel[1], M. G. Chown[2], R. G. Thakar[3], L. Peng[1], T. Desai[1,3], S. Kumar[1]; [1]UCSF/UC Berkeley Joint Graduate Group in Bioengineering, University of California, Berkeley, Berkeley, CA, [2]Department of Bioengineering, University of California, Berkeley, Berkeley, CA, [3]Department of Physiology, Division of Bioengineering, University of California, San Francisco, San Francisco, CA

Regeneration of myocardial tissue through the use of synthetic scaffolds requires strategies to promote cardiomyocyte attachment while minimizing fibroblast proliferation and scar formation. Previous studies have demonstrated that a synthetic platform consisting of an array of microscale polydimethylsiloxane (PDMS)-based pillars ("micropegs") can accomplish both of these goals, yet the mechanism through which micropeg attachment inhibits fibroblast proliferation has remained a mystery. We hypothesize that this suppression of proliferation is related to the ability of fibroblasts to generate high contractile forces against the micropegs, which provide a three-dimensional attachment point for the cells as shown by SEM imaging. We show that when NIH 3T3 fibroblasts are cultured on a micropeg-based scaffold, a cell attached to a micropeg is statistically less likely to proliferate than its counterparts on flat regions of the scaffold; this difference is abrogated when cells are prevented from stressing the micropegs by pharmacologically inhibiting rho-associated kinase (ROCK) and myosin light chain kinase (MLCK). Micropeg adhesion is accompanied by a marked elongation in cell and nuclear shape. We use time-lapse imaging to show that micropegs increase the lengths of tethers formed between the cells and the surface, and that this difference can be abrogated by MLCK inhibitors but not by ROCK inhibitors. These findings support a model in which attachment to the micropegs regulates the ability of the fibroblasts to generate strong contractile forces, correlating with a reduced propensity to proliferate.

1628/B81

Ectopically-Deposited Laminin alpha5 Chain Mediates Adhesion of Hepatocellular Carcinoma through Integrins and/or Lutheran/Basal Cell Adhesion Molecule.

Y. Kikkawa[1,2], R. Sudo[4], J. Kon[2], T. Mizuguchi[3], M. Nomizu[1], K. Hirata[3], T. Mitaka[2]; [1]Laboratory of Clinical Biochemistry, Tokyo University of Pharmacy and Life Sciences, Hachioji, Japan, [2]Department of Pathophysiology, Cancer Research Institute, Sapporo Medical University School of Medicine, Sapporo, Japan, [3]Department of Surgery I, Sapporo Medical University School of Medicine, Sapporo, Japan, [4]Center for Life Science and Technology, Keio University, Yokohama, Japan

Laminins are a diverse group of alpha/beta/gamma heterotrimers formed from five alpha, three beta and three gamma chains; they are major components of all basal laminae (BLs). One laminin chain that has garnered particular interest due to its widespread expression pattern and importance during development is laminin alpha5. Little is known, however, about the expression and function of laminins containing the alpha5 chain in human hepatocellular carcinoma (HCC). Here, using a specific antibody, we examined the expression of laminin alpha5 in normal liver and in HCCs. In normal liver, although laminin alpha5 was observed in hepatic BLs underlying blood vessels and bile ducts, it was absent from the parenchyma, which may be the origin of HCC. On the other hand, laminin alpha5 deposition was observed throughout

all HCCs tested, regardless of tumor grade. In well differentiated HCCs, it localized along the trabecules of the tumor. In poorly differentiated HCCs, it was present in surrounding tumor nodules. In HCC cell lines, laminin alpha5 heterotrimerized with beta and gamma chains and was secreted into the culture media. To attempt to understand the function of laminins containing alpha5, the expression of its receptors in HCCs was also determined. Of the laminin receptors for the alpha5 chain, Lutheran/basal cell adhesion molecule (Lu/B-CAM) and alpha3beta1/alpha6beta1 integrins were variably expressed in HCCs. These receptors were also co-localized with ectopically deposited laminin alpha5 in HCCs, suggesting that they mediate adhesion of HCC cells to the alpha5 chain. In vitro studies showed that HCC cells readily attached to laminin containing the alpha5 chain, more so than did primary hepatocytes. In addition to alpha3beta1/alpha6beta1 integrins and Lu/B-CAM, laminin alpha5 was recognized by integrin alpha1beta1, which was expressed in normal hepatocyte and HCC cells. These results suggest that integrin alpha1beta1 also serve as a receptor for laminin alpha5 in HCCs.

1629/B82

Heparin Binding Sequences in the Laminin A5 Chain LG4-5 Module.

Y. Uchiyama[1], N. Suzuki[2], K. Hozumi[1], Y. Kikkawa[1], M. Nomizu[1]; [1]Tokyo University of Pharmacy and Life Sciences, Tokyo, Japan, [2]National institutes of Health, Bethesda, WA

Laminins are major component of basement membrane and consist of α, β, and γ chain. Laminin α chain has various biological activities. The activities are expressed by interacting with various cell surface receptors including heparan sulfate proteoglycans and integrins. The C-terminal globular domain of laminin α chain consists of 5 LG modules (LG1-5) and plays a critical role in the biological activities of laminins. The α5 chain is expressed at higher levels in adult tissues of lung, heart, bone marrow, pancreas, and kidney. Here, we focused on the laminin α5 chain LG4-5 modules and prepared a recombinant α5 chain LG4-5 protein (rec-α5LG4-5). The rec-α5LG4-5 strongly bound to heparin compared with other α chain LG4-5 proteins. Next, we prepared 43 overlapping synthetic peptides covering the entire the α5 chain LG4-5 module and examined the inhibitory effect on the heparin binding of the protein. 4 peptides, A5G71 (GPLPSYLQFVGI), A5G77 (LVLFLNHGHFVA), A5G81 (AGQWHRVSVRWG), and A5G94 (KMPYVSLELEMR), inhibited the heparin binding of rec-α5LG4-5 in a dose-dependent manner. Active core sequences of the peptides for heparin binding were A5G71 (GPLPSYLQFVGI), A5G77c (LVLFLNHGHFV), A5G81 (AGQWHRVSVRWG), and A5G94b (PYVSLELEMR). These results suggest that heparin binding peptides of α5LG4-5 required at least 10 amino acid sequences. Previously, we identified 6 heparin binding sequences from the α1 - α4 chain LG4-5 modules (AG73, AG75, A2G78, A3G75, A4G82 and A4G83). The α5 chain LG4-5 module has a larger number of the heparin binding peptides compared with that of the other α chains, suggesting that the α5 chain LG4-5 module is highly involved in heparan sulfate proteoglycan mediated biological functions and the number of heparin binding peptide relates to strength of heparin binding for α chain LG4-5 module. These heparin binding peptides are useful for investigating the mechanism of biological activity of the laminin α5 chain.

1630/B83

Hepatocyte Binding Sequences in Laminin Alpha1 Chain.

Y. Matsuda, N. Takahashi, M. Nomizu, Y. Kikkawa; Tokyo University of Pharmacy and Life Sciences, Hachioji, Japan

Laminin-111, a glycoprotein in basement membranes, is a major component of Matrigel. Cell adhesion to laminin-111 regulates cellular behavior such as proliferation, differentiation, migration, and survival. In culture of primary rat hepatocyte, laminin-111 is often used as cell adhesive protein to maintain hepatic functions. Laminin-111 is composed of three subunits, alpha1, beta1, and gamma1 chains. Of three laminin type chains, alpha1 chain plays pivotal roles in cell adhesion. Previously, we have identified 34 cell adhesive sequences in the mouse laminin alpha1 using 321 synthetic peptides. In this study, we screened hepatocyte adhesive peptides using the 34 cell adhesive peptides to examine the maintenance of hepatic function. Rat hepatocytes were isolated by two-step collagenase perfusion and used for cell adhesion assay. When we tested the synthetic peptides in a hepatocyte adhesion assay, 19 peptides revealed the cell adhesion activity in dose dependent manner. Of the peptides, A13 (RQVFQVAYIIIKA), mouse laminin alpha1 chain residues 121-133 exhibited the strongest activity. Furthermore, although the hepatocytes on non-coated plate rapidly lost the expression of metabolic genes, the cells on plate coated with A13 maintained the gene expression such as Tyrosine aminotransferase, Tryptophan-2,3-dioxygenase, and Cytochrome P450 (CYP4A3). The results showed that A13 could not only mediate adhesion of hepatocyte but also maintain several hepatic function. Furthermore we determined the active core sequence of A13, using systematically truncated N- and C-terminal peptides. A13a (VFQVAYIIIKA), with a deletion of N-terminal arginine and glutamine from A13, eliminated its hepatocytes binding activity. A13j (RQVFQVAYI), a C-terminal truncated peptide of A13i (RQVFQVAYII), still retained activity, whereas A13k (RQVFQVAY) with a deletion of C-terminal isoleucine from A13j did not show the activity. These results indicate that the nine-amino acid sequence RQVFQVAYI is critical for A13-hepatocytes adhesion activity. The sequence may be useful tool to clarify micro-environment to regulate hepatic function.

1631/B84

Cell Adhesive Peptides-Conjugated Chitosan Membranes Promote Diverse Biological Activities.

D. Otagiri, C. Fujimori, K. Hozumi, Y. Kikkawa, M. Nomizu; Tokyo University of Pharmacy and Life Sciences, Hachioji, Japan

Laminin-111, a basement membrane protein, plays a critical role in diverse biological activities. We have identified various active sequences in laminin-111 using 673 synthetic peptides. Previously, we have demonstrated that several laminin active peptide-conjugated chitosan membranes enhanced the biological activity and promoted cell adhesion in a cell-type specific manner. We also demonstrated that laminin active peptide-conjugated chitosan membrane can deliver cells and is applicable for keratinocyte transferring to wound bed. Here, we prepared various chitosan membranes (1.5 - 1500 ng/mm^2) and conjugated 1.5 nmol/mm^2 of an αvβ3 integrin binding peptide A99a (ALRGDN) and a syndecan binding peptide AG73 (RKRLQVQLSIR). Cell adhesion to A99a-chitosan membranes was depended on the amount of chitosan. When A99a was conjugated on 3 ng/mm^2 of chitosan, the A99a-chitosan membrane promoted the strongest cell adhesion. In contrast, the cell attachment activity of AG73-chitosan membranes was not affected on the amount of chitosan (1.5 - 1500 ng/mm^2). These results suggest that integrin-mediated cell adhesion is sensitive to physiological property of scaffold. The chitosan membrane (3 ng/mm^2) is a useful platform polymer for evaluating the biological activity of peptide. We conjugated 34 active peptides from laminin-111 to a chitosan membrane (3 nmol/mm^2) and tested the biological activity. Most of the peptide-conjugated chitosan membranes showed strong

cell attachment activities. The peptide-chitosan membranes promoted cell spreading, and morphological appearance of the cells on the peptide-chitosan membranes was peptide-specific. Some of the peptide chitosan membranes interacted with syndecans or integrins. Additionally, several peptide-chitosan membranes promoted neurite outgrowth. These peptide-chitosan membranes promote cell-type specific activities and have a potential to use as a biomedical material.

1632/B85

Identification of Biologically Active Peptides from the Laminin Gamma 2 Chain.

Y. Koike, K. Hozumi, Y. Kikkawa, M. Nomizu; Tokyo University of Pharmacy and Life Scinences, Tokyo, Japan

Laminin γ2 chain is mainly located in the epidermal basement membrane and is composed of EGF-like repeats, a globular domain, and a coiled-coil domain. The γ2 chain is easy to undergo specific proteolytic processing and release LEa (EGF-like repeats) and L4, a globular domain. The cleavage of human laminin γ2 chain decreases the cell adhesion activity and increases the cell motility. Additionally, it is reported that the cleaved L4 fragment promotes tumor metastasis and invasion. Furthermore, the γ2 chain is overexpressed in wound edge, invasion front of tumor cells, and budding tumor cells of many human cancer types. The laminin γ2 chain has been regarded as one of the most typical cancer invasion markers. Here, we focused on the globular domain (L4, position 197-381) of the mouse laminin γ2 chain. We synthesized 22 peptides covering the entire domain to screen the biologically active sequences. These peptides were generally 12 amino acids in length and overlapped with neighboring peptides by 4 amino acids. First, we tested cell attachment activity of the peptides on a peptide-coated plastic plate assay and a peptide-conjugated Sepharose beads assay using HT-1080 human fibrosarcoma cells and HaCaT human keratinocyte cells. Two peptides, C2-26 (VSYGQSLSFDYR: 263-274) and C2-31 (GITKTYTFRLNE: 310-321) showed weak cell attachment activity only the peptide coat plate assay. Three peptides, C2-25 (AKFLGNQQVSYG: 255-266), C2-35 (NLTALLIRATYG: 342-353), and C2-37 (GYIDNVTLVSAR: 358-369), promoted strong cell attachment activity on the both plate and bead assays in a dose-dependent manner. Next, cell migration activity was evaluated by a cloning ring assay using HT1080 cells. The C2-35 peptide significantly promoted HT-1080 cell migration. These results suggest that C2-35 is a biologically active sequence in the laminin γ2 chain L4 globular domain and is useful for investigating the laminin γ2 chain related tumor metastasis.

1633/B86

Cyttype III Collagen Expression, Cell Adhesion and Proliferation Induced by Solubilized Eggshell Membrane Based on Cytocompatible Polymer in Human Dermal Fibroblast.

E. Fujita[1], T. Sakurai[2], T. Konno[3], K. Yoshimura[4], K. Ishihara[3], Y. Atomi[5]; [1]Mechano-Informatics, The University of Tokyo, Bunkyo-ku, Japan, [2]Graduate school of Arts and Sciences, The University of Tokyo, Meguro-ku, Japan, [3]Materials Engineering, The University of Tokyo, Bunkyo-ku, Japan, [4]Medicine, The University of Tokyo, Bunkyo-ku, Japan, [5]IR3S, The University of Tokyo, Bunkyo-ku, Japan

Eggshell membrane protein (EMP) is one of many important bio-food- molecules, which have been usually wasted due to the insolubility and the use as the shield to protect the inside in Western countries, otherwise used in itself in powders to expect improvement of various disease conditions in Asian districts including Japan. Progress of life science have revealed the sheath of food is often constituted of various extracellular matrix proteins (ECM), which is essential for cell survival giving space of cell adhesion. Recently development of a novel biomaterial have revealed the feature of bio-molecules with difficulty to analyze. 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer, PMBN, contain monomer with active ester group as a component. The MPC polymers show excellent cytocompatibility, which can prevent cell adhesion and nonspecific activation of cells. Coating the tissue culture dish with PMBN enables us to analyze the adhesion and other physiological activity of the intended cells on solubilized peptides of EMP (sP-EMP). Using PMBN, we studied basic cellular function of sP-EMP, the native insoluble form of which have been used from old era to expect wound healing resulting in possible dermis regeneration. P-EMP bound to the PMBN applied on cell cultured dish brought us success to show proliferation of human dermal cells with the concentration dependent manner. Dynamic properties of dermis cells attaching and dividing through the interaction with P-EMP conjugated to MPC was directly observed by time-lapse microscopic analysis. Adhesion of dermis fibroblast cell form was visualized by rhodamine-phalloidin actin and immunostained by vinculin. Type III collagen essential for keeping cell dynamics (as well as type I collagen analyzed by RT-PCR) increased with dose-dependent manner in the range from 40 to 120 mg/ml of sP-EMP in human dermis fibroblasts interacting with PEMP bound to MPC polymer coated dish. In conclusion, a part of roles of fine and intrinsic subtle but unanalyzed biological environment produced through evolution such as EMP can be clearly shown by the use of combination of difficult bio-molecules to analyze and bio-materials to give clear a solution.

Cell-Cell Interactions (1634 – 1657)

1634/B87

A Novel Proteolytic Processing of Talin Is Regulated by Arginylation and Associated with Cell-Cell Adhesions.

F. Zhang, A. Kashina; Animal Biology, University of Pennsylvania, Philadelphia, PA

Talin is an essential protein for cell motility and adhesion. It is believed to link integrin receptors to the actin cytoskeleton in cell-substrate adhesions, but no role for talin has been reported in cadherin-mediated cell-cell adhesions. Proteolytic cleavage of talin at an N-terminal site (around aa 360) by calpain has been demonstrated to be involved in focal adhesion turnover, however, other types of proteolytic processing of talin in vivo are not known. During our recent screen for proteins posttranslationally arginylated in vivo we identified an arginylation site at Ala1903 of talin, suggesting a previously unknown proteolytic cleavage at this site. Analysis of mouse embryonic fibroblasts (MEF) by Western blotting, immunoaffinity chromatography, and mass spectrometry revealed an existence of a talin-derived 70 kDa proteolytic product corresponding to the C-terminal region of talin and consistent with cleavage at A1903. This cleavage was blocked by calpain inhibitors and was dependent on cell density. Moreover, this cleavage did not occur in arginyltransferase knockout cells that have severely impaired substrate adhesion and cell-cell contacts. Intracellular levels of the corresponding 70 kDa band in wild type MEF were proportional to the cell number per area in confluent cultures; the 70 kDa band was undetectable in scarce cultures where cells form no connections to

each other. Further experiments suggest that generation of the 70Kd talin fragment likely correlates with the development of cell-cell contacts, but not with cell motility or activity in the cell cycle progression. Moreover, recombinant GFP-talin construct corresponding to the 70 kDa talin fragment (1903-end) appears to be targeted to cadiherin-containing cell-cell contacts, suggesting its direct involvement in cell-cell adhesion. These results suggest a previously unknown proteolytic processing of talin regulated by arginylation that generates a fragment that appears to participate in cell-cell contact formation and/or maintenance.

1635/B88

CRTAM a Molecule with Structural Characteristics of the JAM Protein Family Is Involved in Cell-Cell Adhesion in Epithelial Cells.

E. Garay[1], S. Islas[1], E. Canche-Pool[2], V. Ortiz-Navarrete[2], L. Gonzalez-Mariscal[1]; [1]Physiology, Biophysics and Neuroscience, Cinvestav, Mexico D.F., Mexico, [2]Molecular Biomedicine, CINVESTAV, Mexico D.F., Mexico

CRTAM is a type I integral protein that belongs to the Ig superfamily. It exhibits two Ig-like domains in the extracellular region and contains a class I PDZ binding motif at its carboxyl terminal end and thus can be considered a member of the JAM family of proteins. Here we analyze the expression of CRTAM in epithelial MDCK cells. Employing an antibody against hCRTAM we detect that the presence of CRTAM starts bellow the tight junction (TJ) region and proceeds along the whole lateral membrane, partially co-localizing with E-cadherin and desmoplakin. The distribution of CRTAM is not affected by drugs like cytochalasin B that alter the actin cytoskeleton. Instead acrylamide, that disrupts intermediate filaments, abolishes the cell border distribution pattern of CRTAM. Employing a calcium switch assay we observe that CRTAM exhibits a late arrival to the cell borders after E-cadherin, ZO-1, desmoplakin and occludin. The development of transepithelial electrical resistance (TER) triggered by calcium in monolayers treated for 15 min with EGTA, is not affected by the apical administration of the hCRTAM extracellular domain produced as an Ig-Fc fusion protein (soluble-CRTAM) . Instead when soluble CRTAM is administered to the apical surface of a monolayer transferred to normal calcium media after an overnight incubation in low calcium media, the development of TER is significantly diminished. Thus suggesting that in order to reach its target, soluble CRTAM requires a completely opened paracellular pathway. The administration of CRTAM antibody to sparse cultures of cells one hour after plating, inhibits the formation of cell-cell contacts. Taken together these results suggest that CRTAM is a protein present at the lateral membrane of epithelial cells involved in cell-cell adhesion.

1636/B89

Tubulobulbar Complexes Are Intercellular Podosome-Like Structures That Internalize Intact Intercellular Junctions during Epithelial Remodeling Events in the Testis.

J. S. Young[1], M. De Asis[1], J. A. Guttman[2], A. Vogl[1]; [1]Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada, [2]Biological Sciences, Simon Fraser University, Burnaby, BC, Canada

Tubulobulbar complexes are actin-related double-membrane projections that form at intercellular junctions in the seminiferous epithelium of the mammalian testis. They are proposed to internalize intact junctions during sperm release and during the translocation of spermatocytes through basal junction complexes between neighboring Sertoli cells. The structures resemble podosomes that form at cell/matrix attachments in some other systems and contain N-WASP (WASL), Arp2/3 (ARP2/3), cortactin (CTTN) and dynamin (DNM). In this study, we probe apical tubulobulbar complexes in fixed epithelial fragments and fixed frozen sections of rat and mouse testes for junction molecules reported to be present at apical sites of attachment (ectoplasmic specializations) between Sertoli cells and spermatids. The adhesion molecules nectin 2 (PVRL2), nectin 3 (PVRL3) and α6 integrin (ITGA6) are present in the elongate parts of tubulobulbar complexes and are concentrated at their distal ends. Vesicles at the distal ends of tubulobulbar complexes that contain junction molecules are related to early endosome antigen 1 (EEA1). Our results are consistent with the conclusion that tubulobulbar complexes are podosome-like structures that are responsible for internalizing intact intercellular junctions during spermatogenesis. (NSERC grant #RGPIN 155397-08 to AWV)

1637/B90

P38 MAP Kinase Regulates Cell-Cell Interaction in MCF7 Cells.

Y. Fukui[1], S. Ihara[2]; [1]Hoshi University, Tokyo, Japan, [2]University of Tokyo, Tokyo, Japan

Epitherial cells exhibit intimate cell-cell interaction through the tight junction, the adherens junction, or desmosomes. However, how maintenance of the tight junction and the adherens junctions is regulated is not clearly known. p38 MAP kinase is a member of MAP kinase cascade which transduce cell signaling such as stress responses. We have shown that p38 MAP kinase plays a role in signet ring cell carcinomas, which have lost the cell-cell interaction, suggesting that p38 MAP kinase has an additional role in cell responses. We therefore tested if activation of p38 MAP kinase was involved in cell-cell interaction in a breast cancer cells, MCF7. A cell line, in which expression of constitutively activated MKK6 gene was induced by Cre-loxP system, was generated and tested for the effect of activation of p38 MAP kinase. MCF7 cells grow as aggregates, having tight and adherence junctions. These junctions were lost after induction of the activated MKK6 gene for 36 hrs, resulting in dissociation of the cells from the aggregates. Actin fibers at the junctions appeared to be lost prior to disappearance of occludin or E-cadherin at the cell junctions, suggesting that the signals for dissociation of the cells reach actin before these molecules at the junctions. SB203580, a p38 MAP kinase inhibitor, reversed the dissociation of the cells to form the aggregates. Occludin and E-cadherin assembly was observed within 2 hrs followed by formation of the actin structures. These results suggest that activity of p38 MAP kinase regulates formation of the tight junction and the adherens junction.

1638/B91

Crosstalk between Desmosome-Like and Tight Junctions in the Testis.

P. Lie[1,2], D. D. Mruk[1]; [1]The Population Council, New York, NY, [2]The University of Hong Kong, Hong Kong, China

One of the best-studied phenomena in cell biology is the adhesion of cells to form an organized epithelium, which is facilitated by adherens junctions and desmosomes. In the testis, morphological studies in the 1970s reported the presence of 'desmosome-gap' junctions (D-GJs, also known as desmosome-like junctions) between Sertoli cells (SC) at the blood-testis barrier (BTB), as well as between SC and germ cells (GC). Unfortunately, there is no report addressing the biology of desmosomes and their significance in spermatogenesis. This study aims to fill this

gap by investigating the role of D-GJs in the dynamics of the BTB, whose function is also constituted by tight junctions and ectoplasmic specializations. A survey by RT-PCR revealed that SC and GC express desmosomal proteins, including desmoglein-2, desmocollin-2, plakophilins and desmoplakin. When SC were cultured at high density in vitro, thereby forming a polarized epithelium with functional cell junctions reminiscent of the BTB in vivo, desmosome-like structures were visible by electron microscopy. This coincided with an up-regulation in the levels of several desmosome proteins, illustrating that the testis is equipped with the necessary 'building blocks' to establish functional D-GJs in vivo. Moreover, co-immunoprecipitation experiments using SC lysate revealed that desmoglein-2 associates with plakoglobin and c-Src. To address the role of D-GJs in BTB function, we employed RNAi to specifically knockdown desmoglein-2 in SC in vitro. Besides desmoglein-2, the steady-state levels of JAM-A and ZO-1 decreased when assessed by immunoblotting, but significant changes in the levels of other proteins known to localize to the BTB such as occludin and N-cadherin were not detected. These findings were consistent with results using immunofluorescence microscopy which illustrated a loss of ZO-1 from sites of Sertoli cell contact following desmoglein-2 RNAi. Additional studies are now underway to determine if mislocalization of ZO-1 can perturb BTB function.

1639/B92

Expression of Multiple Cadherins in a Cancer Co-Culture Model System.

M. Apostolopoulou, G. Quinones, R. Dash, L. Ligon; Rensselaer Polytechnic Institute, Troy, NY

In the early stages of breast cancer metastasis, epithelial tumor cells penetrate the basement membrane and invade the surrounding stroma, where they encounter stromal fibroblasts. Recent evidence has suggested that bi-directional biochemical communication between fibroblasts and epithelial tumor cells contributes to the metastatic cascade. Here we are examining the development of adhesive contacts between epithelial tumor cells and fibroblasts to determine the contribution of heterotypic adhesions to the metastatic cascade. Adhesions between epithelial cells typically use E-cadherin and those between fibroblasts typically use N-cadherin, but it is unknown what kind of adhesion molecules are used when these two cell types come in contact with each other. Here we have used RT-PCR to detect the expression of other members of the cadherin superfamily expressed by MCF-7 breast cancer cells and human fibroblasts, both in isolation and when grown together in co-culture. The results of those experiments suggest that both MCF-7 cells and human fibroblasts express a variety of cadherin molecules in addition to E- and N-cadherin, including P- and OB-cadherin. P-cadherin, a molecule normally expressed in the basal cells of the epidermis, was localized by immunocytochemistry at the adhesion sites between MCF-7 cells. Immunocytochemistry experiments were also used to localize OB-cadherin at the adhesion sites between human fibroblasts, but neither P- nor OB-cadherin appear to mediate heterotypic adhesions between MCF-7 cells and fibroblasts. These data confirm the hypothesis that breast cancer cells and human fibroblasts express a variety of cadherin molecules. In addition, our analysis suggests that cadherins 13, 20 and 24, along with desmocollin-2 are expressed by our experimental system, but their role in homotypic or heterotypic adhesions remains to be determined.

1640/B93

Biophysical Approaches to Study Crosstalk between Cell Adhesion Molecules.

C. Martinez Rico[1,4], F. Pincet[2], M. Sheetz[1], J. Thiery[3], S. Dufour[4]; [1]Columbia University, New York, NY, [2]ENS, Paris, France, [3]IMCB, Singapore, Singapore, [4]Institut Curie, Paris, France

Abstract Cadherins and integrins are major adhesion molecules regulating cell-cell and cell-matrix interactions. In vitro and in vivo studies have demonstrated the existence of crosstalk between integrins and cadherins in cell adhesion and motility. Our study provides a mechanistic insight into integrin-cadherin cross-talk and this signaling process will be analyzed in a physical context, using both, laser trap and cell micromanipulation experiments. Results Cell micromanipulation experiments We investigated the role of integrin stimulation in the development of cadherin-mediated adhesion, by stimulating cells with fibronectin-coated beads (FNbead) before allowing them to come into contact for four minutes and measuring the Separation Force (SF). The SF required to separate Ecad cell doublets not bound to FNbeads was 45.9 ± 11.1 nN. The SF for doublets of Ecad cells bound to three 10 µm-FNbeads or one 15 µm-FNbead displayed an increase by a factor of 1.6 in response to integrin-dependent stimulation. Laser trap We used the laser tweezers to quantitatively analyse N-cadherin-mediated adhesion when integrin attachment stimulates the formation of focal adhesion complexes. We have found that the time of contact between N-cadherin expressing cells and N-cadherin-coated bead depends on the formation of mature focal adhesion. Overnight spreading cells on FN requires 61.4±10.3 sec to pull and break the adhesion of the trapped bead. In contrast, 19±3.3 sec of contact were necessary for PL spreading cells,. We have found that spreading cells on FN display higher trap stiffness (26.5±6.4 pN/µm) than PL spreading cells (5.1±0.6 pN/µm). Conclusions This separation force and the trap stiffness were used as an index of intercellular adhesion and were determined under different conditions, to elucidate the mechanisms regulating adhesion strength. Both techniques measure adhesion forces, but each of them measures different levels of force, therefore allowing us to explore in detail the crosstalk between cell-matrix and cell-cell adhesion processes.

1641/B94

Up-Regulation of Semaphorin 3A in Human Corneal Fibroblasts by Epidermal Growth Factor Released from Cocultured Human Corneal Epithelial Cells.

J. Ko, N. Morishige, T. Nishida; Ophthalmology, Yamaguchi University Graduate School of Medicine, Yamaguchi Ube, Japan

Semaphorins are a family of glycoproteins that play an important role in repulsive axon guidance during embryogenesis. We have now investigated the effect of corneal epithelial cells on the expression of Sema3A in corneal fibroblasts with the use of a coculture system in which the two cell types are separated by a collagen vitrigel membrane. Reverse transcription-polymerase chain reaction and immunoblot analyses revealed that the presence of immortalized human corneal epithelial (HCE) cells increased the expression of Sema3A in human corneal fibroblasts at both the mRNA and protein levels. This effect of HCE cells was mimicked by recombinant human epidermal growth factor (EGF) in a concentration- and time-dependent manner. An inhibitor of the tyrosine kinase activity of the EGF receptor, PD153035, blocked the EGF-induced up-regulation of both Sema3A mRNA and protein in corneal fibroblasts. Depletion of EGF in HCE cells by RNA interference largely abolished the effect of these cells on Sema3A expression in corneal fibroblasts. These findings indicate that EGF released from corneal epithelial cells up-regulates the expression of Sema3A in corneal fibroblasts. This effect of EGF may play an important role in maintenance of corneal structure and repair of corneal damage.

1642/B95

Understanding Cellular Reorganization in a Dynamic Epithelium, the Amnioserosa, during Drosophila Dorsal Closure.

C. Meghana[1], N. Ramdas[2], G. V. Shivashankar[2], M. Narasimha[1]; [1]Dept. Of Biological Sciences, Tata Institute Of Fundamental Research, Mumbai, India, [2]National Centre For Biological Sciences, Tata Institute of Fundamental Research, Bangalore, India

Epithelial morphogenesis is accomplished by the dynamic reorganization of cells. The molecules and mechanisms that mediate reorganization and contribute to tissue geometries still remain elusive. Cell adhesion molecules regulate both physical and chemical connectivity between cells in epithelia and influence epithelial morphogenesis which has also been shown to be governed by topological cues and mechanical forces. We have explored the utility of the amnioserosa, a highly dynamic extra embryonic tissue that participates in Drosophila dorsal closure as a model to study adhesion dependent cellular reorganization. We have used sub cellular laser ablations to understand the nature of the physical constraints operating on cells in a dynamic epithelium. Our results identify laser perturbations as an attractive tool to understand the influence cell mechanics in an active epithelium.

1643/B96

The Regulation of Cell Fusion in Neurospora Crassa.

A. C. Leeder[1], A. Fleissner[2], A. R. Simonin[1], N. L. Glass[1]; [1]Plant & Microbial Biology, University of California, Berkeley, Berkeley, CA, [2]Institut fur Genetik, Technische Universitaet Braunschweig, Braunschweig, Germany

Vegetative cell fusion between self-self pairs of fungal conidia has long been observed, and the process is integral to the efficient formation of a hyphal network. These fusions are thought to have many benefits, including the sharing of nutrient resources. How these cells detect one another is currently unknown, but we describe new insights into the regulation of the chemotropic interactions that are crucial to the conidial fusion process. MAK-2, a homologue of *S. cerevisiae* Fus3p, is essential for vegetative fusion in *Neurospora crassa*. It was previously shown that MAK-2-GFP localizes to tips of germlings engaged in chemotropic attraction. Results indicate interdependence between the kinase activity and localization of MAK-2. Data also suggest a role for MAK-2 in the regulation of another *N. crassa* protein SO. SO, whose molecular function is unknown, is also required for cell fusion and localizes to hyphal tips involved in this process. The MAPK cascade that includes MAK-2 is also thought to be required for sexual cell fusion in *N. crassa*. Using localization studies and mutant alleles, we show a difference in the requirement for MAK-2 during sexual cell fusion as compared to vegetative fusion.

1644/B97

Intercellular Transfer to Signaling Endosomes for Targeted Regulation within a Bone Marrow Niche.

J. Gillette, J. Lippincott-Schwartz; NICHD, CBMB, National Institutes of Health, Bethesda, MD

Cellular communication within a larger microenvironment is critical for a number of physiological processes. Within the bone marrow niche, direct cell communication between hematopoietic progenitor cells (HPCs) and osteoblasts provides essential cues for their proliferation and survival. While contact-dependent communication between HPCs and osteoblasts is known to be critical, the molecular pathways that govern this interaction are largely unclear. Moreover, the downstream events occurring at the HPC/osteoblast contact site remain uncharacterized, despite their major role in signaling and remodeling within the niche microenvironment. Using live cell imaging approaches, we have found that intercellular transfer is a novel mode of cell communication within the bone marrow niche microenvironment. HPCs made prolonged contact with the osteoblast surface via a specialized membrane domain enriched in prominin 1, CD63 and rhodamine PE. At the contact site, portions of the HPC specialized domain containing these molecules were taken up by the osteoblast and internalized into long-lived, SARA-positive, signaling endosomes. This resulted in the downregulation of Smad signaling by the osteoblasts and a subsequent increase in the production of stromal-derived factor-1, a chemokine responsible for HPC homing to bone marrow. These findings identify a novel mechanism involving intercellular transfer to signaling endosomes for targeted regulation of signaling and remodeling events within the osteoblastic niche microenvironment.

1645/B98

Expression of an Engineered Gs-coupled Receptor, Rs1, in Mature Osteoblasts, In Vivo, Drives Expansion of Early Osteoblast-Lineage Cells and Osteoclasts.

S. M. Millard[1], A. Louie[1], B. Boudignon[1], W. Lu[1], B. Halloran[1], B. Conklin[2], R. Nissenson[1]; [1]Endocrine Research Unit, VA Medical Center, San Francisco, CA, [2]Gladstone Institutes, University of California, San Francisco, CA

The structure and volume of bone at a given site is determined by the activity of bone forming osteoblasts and bone resorbing osteoclasts. The activities of these cells are influenced by a complex array of signals and cell-cell interactions. We have examined the role of Gs-coupled signaling pathways in osteoblasts using targeted expression of an engineered constitutively active Gs-coupled receptor, Rs1. Expression of Rs1 in maturing and mature osteoblasts in vivo using the Collagen Iα 2.3 kb promoter was previously shown by us to induce a dramatic anabolic skeletal response, including a striking expansion of what appear to be early osteoblast lineage cells. Conceptually, these cells may be derived either from an expansion of Rs1 expressing cells, which fail to fully differentiate, or from osteoblast progenitors that can respond to paracrine signals generated by Rs1 expressing cells. To investigate this question, we have expressed Rs1 under the control of the osteocalcin promoter, to restrict transgene expression to mature osteoblasts. MicroCT of femurs from OC:Rs1 mice showed a massive and generalized increase in mineralized bone by 9 weeks of age. Histomorphometry showed a marked increase in bone cellularity and disorganized trabecular bone. Expression of Runx2 and alkaline phosphatase (abundantly present in early osteoblast lineage cells) were both upregulated >5 fold in RNA isolated from tibiae of 9wk old OC:Rs1 animals. Osteocalcin expression (restricted to mature osteoblasts) was only modestly upregulated (2 fold). Gs-dependent cAMP signaling is known to increase expression of the osteoclastogenic factor, RANKL, by osteoblasts. Accordingly, RNA levels of RANKL were found to be upregulated by 7 fold, with the ratio of RANKL to its decoy receptor, OPG, increasing by 2 fold. Histomorphometry revealed a striking increase in positive staining for the osteoclast marker, TRAP, in bones of OC:Rs1 mice. Thus, Gs signaling in mature osteoblasts drives a large increase in bone formation and bone resorption. The former appears to result from paracrine control of the number of early osteoblasts whereas the latter likely arises from a Gs-induced increase in the osteoclastogenic activity of mature osteoblasts.

1646/B99

Schwann Cell Behavior and Phenotypes in a Model of Autologous Nerve Graft.

I. Alcalde[1,2,3], S. Del Olmo Aguado[3], M. Gayoso[1,2], I. De la Fuente-Ayuso[2], R. Cantalapiedra[3], I. Fernandez-Bueno[3]; [1]INCYL, University of Valladolid, Valladolid, Spain, [2]Dpt. of Cell Biology and Histology, University of Valladolid, Valladolid, Spain, [3]IOBA, University of Valladolid, Valladolid, Spain

The gold standard in a severe sectioned peripheral nerve repair is the autologous graft. This graft represents a favorable environment that allows nerve regeneration. Schwann cells provide axons a basal lamina and numerous trophic factors. Nevertheless, little is known about interactions between engrafted tissue cells and growing axons from the proximal stump. Objective: To study the behavior and the relationship of donor Schwann cells with host's regenerating axons. Methods: 1 cm sciatic nerve segments were collected from EGFP mice and inserted into a similar gap in nu/nu mice (immunosupressed). After survival periods of 1, 2 and 4 weeks, engrafted nerves were removed and processed for immunofluorescence techniques. We used S100, GFAP and MBP markers to demonstrate Schwann cell phenotype, and Neurofilaments to show regenerating axons. Animals were handled and cared following the guidelines of the European Commissions (86/609/EC). Results: As early as 1 week after surgery we observed the typical Wallerian degeneration features inside the graft as well as in the host's distal stump. Donor Schwann cells proliferate largely and begin to associate with axons from proximal stump. Some of these cells migrates invading host's tissue both into distal and proximal stumps. There is a great increase in number of amyelinic phenotype fibers at this stage. Later, most of these fibers become myelinated, showing MBP marker and establishing a permanent 1:1 relation between Schwann cells and axons. Conclusions: Therefore engrafted Schwann cells play an active role in myelination of regenerating axons, not only supporting them with neurotrophic factors but enwrapping and myelinating the axons even in the host tissue environment.

1647/B100

Thy-1/αVβ3 Integrin Interaction Triggers Bi-Directional Signaling between Neurons and Astrocytes.

R. A. Herrera-Molina, A. F. Quest, L. Leyton; Cell and Molecular Biology, Facultad de Medicina, Universidad de Chile, Santiago, Chile

Thy-1 is a highly expressed neuronal glycoprotein, of poorly defined function that is implicated in neurite outgrowth inhibition. Our findings indicate that αVβ3 integrin is a receptor for Thy-1 on astrocytes, which, upon engagement, triggers integrin clustering, tyrosine phosphorylation and activation of RhoA, thereby increasing astrocyte adhesion. Here, we evaluated whether αVβ3 integrin on astrocytes acts also as a Thy-1 ligand inducing inhibition of neurite outgrowth. To this end, neurite outgrowth was induced by serum deprivation of CAD cells (Cath-derived cell line) seeded over DITNC1 cells (astrocyte cell line), the fusion protein αVβ3-Fc-coated plates or control plates. Additionally, the bi-directionality of the Thy-1/αVβ3 integrin interaction was visualized by 3D imaging of fluorescently labelled focal adhesions formed in astrocytes upon contact with neurons. Visualization of the cell contact area in the xz or yz planes revealed that focal adhesions formed around the area of neuron astrocyte association. Interestingly, antibodies against Thy-1 inhibited formation of focal adhesions but not the binding of neurons to astrocytes. On the other hand, neuronal cells seeded over an αVβ3-Fc matrix developed fewer and shorter neurites compared to those seeded over the control matrix. Moreover, when CAD cells were seeded over DITNC1 cells —but not over DITNC1 cells pre-treated with Thy-1-Fc protein or antibodies against αV or β3 integrins— neurite outgrowth was also inhibited. Therefore, although many molecules mediate neuron-astrocyte communication, the changes described here in astrocytes depended solely on the Thy-1/αVβ3 integrin interaction. Additionally, αVβ3 integrin expressed by DITNC1 cells was responsible for inhibition of neurite outgrowth. Thus, Thy-1/αVβ3 integrin interaction represents a novel mechanism of bi-directional cell communication between neurons and astrocytes that triggers morphological changes in both cells. Ph.D. CONICYT fellowships (R H-M), FONDAP #15010006 (AFGQ), FONDECYT #1070699 (LL).

1648/B101

Acute Low-Dose Arsenic Alters P2 Receptor-Dependent Ca2+ Signaling in Human Airway Epithelial Cells.

S. Boitano[1,2,3], C. L. Sherwood[1,3,5], A. E. Liguori[1,4,5], C. E. Olsen[1,4,5], J. L. Burgess[4,6], R. Lantz[3,4,5]; [1]Arizona Respiratory Center, Arizona Health Sciences Center, Tucson, AZ, [2]Physiology, Arizona Health Sciences Center, Tucson, AZ, [3]Cell Biology and Anatomy, Arizona Health Sciences Center, Tucson, AZ, [4]Southwest Environmental Health Sciences Center, University of Arizona, Tucson, AZ, [5]Bio5 Research Institute, University of Arizona, Tucson, AZ, [6]Mel & Enid Zuckerman College of Public Health, University of Arizona, Tucson, AZ

As part of the innate immune defense system, the polarized conducting airway epithelium regulates salt and water transport and acts as a barrier to keep particulates carried in respiration from underlying tissue. Arsenic is a metalloid toxicant that can affect the lung via inhalation or ingestion. We have recently shown that acute low-dose arsenic exposure inhibits wound repair in part through up-regulation of MMP-9 in human lung epithelial cells. In this report we use an *in vitro* model to examine effects of acute exposure of low-dose arsenic (0, 60, and 290 ppb) on Ca^{2+} signaling, another component that contributes to wound recognition/repair as well as to salt and water transport in the airway epithelium. We found that 24 hr exposure to arsenic results in a reduction in nucleotide-dependent intercellular Ca^{2+} signaling emanating from a scrape wound. Using a localized wound model (1-2 cells) we showed that the observed arsenic-induced loss of Ca^{2+} response in cells immediately adjacent to the wound is dose-dependent. Loss of Ca^{2+} signaling at the lower doses (24 hr exposure to 60 ppb arsenic) was primarily a consequence of a more rapid recovery of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) to resting values. Loss of Ca^{2+} signaling at higher doses (24 hr exposure to 290 ppb arsenic) was a result of a reduced number of cells with a Ca^{2+} response and a reduction in peak $[Ca^{2+}]_i$ change in those cells that did respond. In an effort to uncover molecular mechanisms that underlie the observed changes in Ca^{2+} signaling, we examined P2X and P2Y receptors reported to be expressed in human airway epithelium. We found that at the 60 ppb arsenic dose, $P2X_4$ and/or $P2X_5$ receptor signaling is altered, whereas $P2Y_2$ receptors are down-regulated following 290 ppb arsenic exposure. We propose that arsenic in the airway can alter paracrine P2 receptor signaling in the epithelium and this change has direct effects on wound recognition/repair as well as on salt and water transport.

1649/B102

Crosstalk between Cell-Cell Adhesion and Cell Migration at the Single Cell Scale Studied by Dual Substrate Micropatterning.

N. Borghi[1], J. Nelson[1,2]; [1]Department of Biology, School of Humanities and Sciences, Stanford University, Stanford, CA, [2]Department of Molecular & Cellular Physiology, School of Medicine, Stanford University, Stanford, CA

The balance between cell-cell adhesion and cell migration is of critical importance for the normal development of multicellular organisms, and disruptions in this balance lead to loss of tissue cohesion as observed in metastatic cancers. Sedentary and migratory cell behaviors result from the integration of intercellular adhesion and extracellular matrix adhesion cues, but the underlying intracellular mechanisms lack a systematic and quantitative understanding. Cellular responses to extracellular matrix adhesion signaling have been widely investigated using surface micropatterning. We have expanded this technique to create alternating micropatterns of the extracellular matrix protein Collagen IV and the cell-cell adhesion protein E-cadherin on which we monitor normal epithelial cell (Madin-Darby Canine Kidney) behavior at multiple space and time scales by fluorescence live-cell microscopy. By choosing pattern geometries that allow a single cell to contact multiple patterns, we are able to elicit different adhesion responses in distinct zones of a single cell. Several outputs can be measured simultaneously at the single cell level in response to varying levels of adhesive surface proteins. We find that recruitment of GFP-tagged cadherin/catenin- and integrin-mediated adhesion proteins is specific and localized to distinct adhesion patterns: E-cadherin, β-catenin and α-catenin are recruited on E-cadherin patterns in a concentration dependent manner, whereas focal adhesions, as visualized with vinculin, are restricted to collagen patterns. Cytoskeleton organization and lamellipodial activity are also specific to adhesion patterns with a disrupted actin cortical bundle and a dampened lamellipidial activity on E-cadherin patterns compared to collagen patterns. In turn, specific features of cell migration, such as directionality but not rate, are affected. This experimental approach offers new and novel insights into the relationship between typical features of intercellular and cell-matrix adhesion and their contributions to cell signaling, cytoskeletal dynamics, and migration, all of which are required to regulate tissue cohesion.

1650/B103

Interaction between Cisplatin Treated Murine Peritoneal Macrophages and L929 Cells: Involvement of Adhesion Molecules, Cytoskeletons, Upregulation of Ca2+ and Nitric Oxide Dependent Cytotoxicity.

P. Chauhan, A. Sodhi; School of Biotechnology, Faculty of Science, Banaras Hindu University, Varanasi, India

Murine peritoneal macrophages on treatment with cisplatin (10 µg/ml) showed 50-60% increased binding to L929 cells. DIC and Immunofluorescence microscopic studies showed that cisplatin treated macrophages on co-incubation with L929 cells form a distinct cytoplasmic contact between the two cells. The plasmalemmae of the two cells fuse over a large surface area. The formation of contact between the cisplatin treated macrophage and L929 cell results in the induction of apoptosis in L929 cell. Untreated macrophages did not form a contact with L929 cells and no apoptosis is observed in L929 cells. Immunofluorescence microscopical studies clearly show the participation of cytoskeleton and the adhesion molecules (LFA-1 and ICAM-1) in the formation of contact between the two cells. Further, a significant enhancement of the expression of iNOS and cytosolic Ca2+ was observed in cisplatin treated macrophages co-incubated with L929 cells. Cisplatin treated macrophages produced significant amount of NO when co-incubated with L929 cells (40µM), while there was minimal production of NO by untreated macrophages co-incubated with L929 cells (10µM). Cisplatin treated macrophage showed 68-70% cytotoxicity against L929 cells. L929 cell death induced by cisplatin treated macrophages was NO dependent, since L-NMMA (500µM) significantly inhibited the cytotoxicity of L929 cells by 24%. The addition of excess L-arginine (2mM) reversed the L-NMMA induced inhibition of NO production and L929 cell cytotoxicity. The present investigations, therefore, suggest that cisplatin treated macrophages co-incubated with L929 cells, results in the formation of a cytoplasmic contact between the two cells. The cell membranes, adhesion molecules and the cytoskeleton participate in the formation of this contact, which finally results in the L929 cell death through apoptosis mediated by nitric oxide produced by macrophages.

1651/B104

Novel Candidates to Mediate Homotypic and Heterotypic Endothelial Cell Interactions during the Human Inflammatory Response.

N. Reglero[1], S. Gharbi[3], M. A. Alonso[1], A. J. Ridley[2,3], J. Millán[1,3]; [1]Departamento de Biología Molecular, Centro de Biología Molecular Severo Ochoa. Universidad Autónoma de Madrid, Madrid, Spain, [2]Randall Division of Cell and Molecular Biophysics, King's College London, London, United Kingdom, [3]Ludwig Institute for Cancer Research, London, United Kingdom

The endothelium responds to long term inflammation changing shape and modulating gene expression in order to increase its permeability to small molecules and cells from the bloodstream. Endothelial permeability to both molecules and cells constitutes one of the first defences against infection but it also underlies the pathogenesis of many important chronic diseases such as atherosclerosis, multiple sclerosis or rheumatoid arthritis. In addition, proinflammatory stimuli such as TNF-alpha have been reported as pro- or anti-angiogenic depending on the model and/or context of the studies. This is suggestive of a link between inflammation and tumourogenesis still not well understood. We have hypothesized that those proteins that play important roles during inflammation must be expressed and localized at the surface on endothelial cells in response to proinflammatory stimuli. We have performed a proteomic and quantitative PCR screening of novel proteins transiently localized at the apical membrane of human primary endothelial cells in response to TNF-alpha. We have found new candidates to regulate leucocyte transendothelial migration, endothelial cell-cell junctions, such as cadherins and protocadherins, and endothelial cell migration during long term inflammation. In this latter group we have found receptors that play an important role in axonal guidance, such as jagged-1, which suggest common clues between neural development and the endothelial inflammatory response.

1652/B105

Rosettes between Human Blood Monocyte-Derived Macrophages and Lymphocytes in Samples Harvested from Human Mixed Cultures.

I. Novak, H. Cabral; Facultad de Ciencias Medicas, Instituto de Biologia Celular, Universidad Nacional de Cordoba, Cordoba, Argentina

Blood monocyte-derived macrophages and lymphocytes, when harvested from cell cultures of total human leukocytes, showed selective cell-cell interactions and the ability to form rosettes and immunological synapses between them, and a linear relation between such rosettes formation and culture-time occurs, as we reported previously (Rev Fac Cienc Med Cordoba 50 (2):25-6, 1992; Mol Biol Cell 7:S, 603a, ASCB 1996; Mol Biol Cell 8:S, 414a, ASCB 1997; Am J Hematol 60: 285-8, 1999; Mol Biol Cell 16:S 108-109a, ASCB 2005). The rosettes

are formed by a central macrophage surrounded by three or more T CD4 lymphocytes (Am J Hematol 60: 285-8, 1999). We postulated that antigen presentation would be involved in such rosettes and we found that CD66 monoclonal antibodies recognize neutrophil material phagocitized by macrophages that formed immunological synapses and rosettes (Mol Biol Cell 13:S, 393a, ASCB 2002). Here, we study the formation of rosettes between human blood monocyte-derived macrophages and lymphocytes in samples harvested from human mixed total leukocyte cultures. In this model the immune response is allogeneic. Methods: homologous human total leukocyte cultures from 9 healthy donors. Samples were harvested at various times: 24, 48, 72, 96 and 120 hours of culture and centrifuged and performed as previously reported. Results are expressed as the mean of % number of macrophages that form rosettes, considering 100 of such cells in the respective cyto-preparations. Results: in all the cases studied, the formation of rosettes between co-cultured blood monocyte-derived macrophages and lymphocytes, was confirmed. The mean number of rosettes at 24 hours was 3%, at 48 hours was 5%, at 72 hours was 6%, at 96 hours was 10% and at 120 hours was 13%. Also in the same cyto-preparations we found big cells like blasts interspersed. Conclusions: Rosettes between human blood monocyte-derived macrophages and lymphocytes in human mixed cultures were observed.

1653/B106

Conserved Function of Shroom-Family Proteins in the Regulation of Epithelial cell morphology.

J. Hildebrand; Biological Sciences, University of Pittsburgh, Pittsburgh, PA

The Shroom family of proteins are key participants in several developmental and adult processes, including neural tube morphogenesis (Shroom3), retina formation (Shroom2), and behavior (Shroom4), that function at the level cytoskeletal organization and cellular morphology. These proteins all exhibit the capacity to directly bind F-actin in vitro and colocalize to distinct compartments of F-actin in vivo. Shroom proteins contain a C-terminal Shroom-domain 2 (SD2) that is both necessary and sufficient to induce changes in cell morphology. SD2 functions to regulate the subcellular distribution of Rock, resulting in the spatial regulation of myosin II activity. This is best characterized for Shroom3, which localizes to the apical junctional complex in polarized epithelia and recruits Rock to this location. This facilitates the formation of a contractile acto-myosin network in the apical region of the cells which functions to control either apical constriction or apical tension in the neural epithelium. We have used a genetic approach to investigate the role of Shroom3 in other aspects of vertebrate neural tube morphogenesis. Through this approach we have determined that there is a genetic interaction between Shroom3 and the planar cell polarity pathway. The link between these pathways occurs at the level of Dishevelled, as Shroom3 and Dishevelled co-distribute in cells and this localization can be regulated by Frizzled. To further our understanding of how Shroom proteins function, we have also characterized the Drosophila ortholog of Shroom and are utilizing this genetic system to further understand the regulators and effectors of Shroom. Our results indicate that dShroom is virtually indistinguishable from mouse Shroom3 in both subcellular distribution and activity in vivo. We propose that Shroom proteins comprise an evolutionarily conserved family of actin-associated proteins that function to regulate epithelial morphology during numerous developmental processes.

1654/B107

The C. Elegans EAT-1 Protein Regulates Adenosine Signaling to Control Feeding Behavior.

H. Han[1,2], M. Beckerle[1,2]; [1]Oncological Sciences Department, University of Utah, Salt Lake City, UT, [2]Huntsman Cancer Institute, Salt Lake City, UT

How cells communicate to regulate basic cellular functions and coordinate diverse activities is one of the central questions in the biology of multicellular organisms. The *C. elegans* pharynx is an excellent model system with a wide variety of genetic tools and quantitative assays to study the cellular mechanisms that underlie rhythmic feeding behavior. The *eat-1* mutants were isolated based on their slow, irregular pharyngeal pumping phenotype. Based on genomic transformation data, RNA interference knockdown, as well mutation identification in two available *eat-1* mutants, we identified that *eat-1* encodes an evolutionarily conserved protein, adenosine kinase. Adenosine kinase participates in purine metabolism and functions to catalyze adenosine to adenosine monophosphate (AMP), however, the biological contributions of adenosine kinase remains poorly understood. To begin an analysis of adenosine kinase in *C. elegans*, we have determined the expression profile of EAT-1 using an EAT-1::GFP fusion protein under the control of its native promoter. EAT-1 is ubiquitously expressed at most of the tissues in the worm, including pharynx, intestine and hypodermis. Using tissue-specific rescue experiments, we showed that expression of EAT-1 in hypodermis specifically rescued *eat-1* pharyngeal pumping defect, indicating that EAT-1 acts non-cell-autonomously to regulate pharyngeal function. Because of the non-autonomous nature of the *eat-1* mutations, we hypothesized that loss of EAT-1 results in accumulation of extracellular adenosine, which then results in misregulation of pharyngeal pumping. To test this idea, we treated dissected pharynxes with adenosine and found pharyngeal pumping is suppressed, demonstrating the specific role of accumulated adenosine in regulation of pharyngeal function. Together, our results reveal the important role of adenosine kinase in cell-cell communication to regulate cellular behavior.

1655/B108

Endothelial SHP2 Is Phosphorylated through Mechanical Stimuli.

B. Krovic, E. McBeath, Y. Chiu, K. Fujiwara; Aab CVRI, University of Rochester School of Medicine and Dentistry, West Henrietta, NY

The Src homology 2-containing protein tyrosine phosphatase (SHP2) is required by cytokine receptors, receptor tyrosine kinases, and integrins for full activation of the ERK pathway and is essential for the shear stress (SS)-induced activation of the protein kinase A-dependant eNOS pathway in endothelial cells. In addition, our laboratory has shown that PECAM-1, a tentative mechanotransducer molecule of endothelial cells (ECs), is necessary for the activation of the ERK pathway by hyperosmotic shock (HOS) and SS. Upon HOS and SS, PECAM-1 is tyrosine phosphorylated and recruits SHP2 to the cell-cell contacts where PECAM-1 is localized. Both the PECAM-1-SHP2 interaction and the phosphatase activity of SHP2 are necessary for ERK activation by these mechanical stresses. To understand how SHP2 function is regulated in mechanically stimulated ECs, we studied SHP2 phosphorylation in ECs stimulated by SS, HOS, and cyclic stretch. We found that SHP2 was tyrosine phosphorylated within minutes under these conditions. This phosphorylation was PP2 sensitive, suggesting the involvement of Src family kinases in this response. We found that the SHP2 coimmunoprecipitated with PECAM-1 was highly phosphorylated. To see if SHP2 phosphorylation was PECAM-1 dependent, we knocked-down PECAM-1 expression by siRNA and found

SHP2 phosphorylation unaffected in these cells. Our studies show that SHP2 is phosphorylated by mechanical stresses in a PECAM-1 independent manner, suggesting that SHP2 phosphorylation is triggered by a mechanosensor different from PECAM-1.

1656/B109

Cis/Trans Competition in Heterotypic Adhesive Signaling: Macrophage CD47 Binds Its Own Cell-surface SIRPa.

R. Tsai, D. Discher; University of Pennsylvania, Philadelphia, PA

The Marker of Self adhesion protein CD47 is found on all cell types and binds SIRPa on Macrophages in trans at sub-microMolar affinities, but human Macrophages also express CD47 and competition in cis proves significant. Binding of CD47 in trans is known to inhibit phagocytosis of CD47-displaying targets (of various diameter targets we will show), and we show here that knockdown of CD47 on Macrophages leads to greater binding of soluble CD47 and greater phagocytosis of opsonized targets. The effective Kd for binding in trans depends linearly on cis CD47 on the Macrophage, consistent with the simplest competition model. Additionally, CHO cells engineered to express CD47-GFP bind more soluble SIRPa than CHO cells displaying both CD47 and SIRPa, although high affinity antibodies can out-compete cis interactions. Both Hematopoietic and Mesenchymal Stem Cells also display both CD47 and SIRPa, but trans interactions on these cells are clearly inhibited. Cis interaction between the lone immunoglobulin (Ig) domain of CD47 and the distal of SIRPa's three Ig domains suggests a generality to cis/trans competition between the many multi-domain membrane adhesion proteins.

1657/B110

IL-6 Loss Causes Ventricular Dysfunction, Fibrosis, Reduced Capillary Density and Dramatically Alters the Cell Populations of the Heart.

I. Banerjee, J. W. Fuseler, A. R. Intwala, T. A. Baudino; CBA, University of South Carolina School of Medicine, Columbia, SC

Heart disease is the leading cause of morbidity and mortality in the United States with approximately 11% of the adult population reporting some type of heart disease. At the cellular level, during cardiovascular pathology and homeostasis, dynamic interactions between cardiac fibroblasts, myocytes and the extracellular matrix (ECM) have been shown to be critical. Interleukin-6 (IL-6) is a pleiotropic cytokine responsible for many different processes including the regulation of cell growth, apoptosis, differentiation and survival in various cell types and organs, including the heart. Recent studies have indicated that IL-6 is a critical component in the cell-cell communication between myocytes and cardiac fibroblasts. Here we examine the effects of *IL-6*-deficiency on the cardiac cell populations, cardiac function and interactions between the cells of the heart, specifically cardiac fibroblasts and myocytes. To examine the effects of *IL-6*-loss on cardiac function, we used the *IL-6*$^{-/-}$ mouse.*IL-6*-deficiency was observed to cause severe cardiac dilatation, increased accumulation of interstitial collagen and altered expression of the adhesion protein periostin. In addition, fluorescence activated cell sorting analysis demonstrated dramatic alterations in the cardiac cell populations of *IL-6*$^{-/-}$ mice when compared to wild type littermates. We observed a marked increase in the cardiac fibroblast population in *IL-6*$^{-/-}$mice, while a concommitment decrease was observed in the other cardiac cell populations examined. Moreover, we observed an increase in both proliferation and apoptosis in the developing *IL-6*$^{-/-}$ heart. Furthermore, we observed a significant decrease in the capillary density of *IL-6*$^{-/-}$ hearts. To examine the mechanism of IL-6's function in the heart, we performed in vitro studies that demonstrated that *IL-6*-deficiency altered cardiac fibroblast-myocyte interactions and affected signaling through the STAT3 pathway. Taken together, these data demonstrate that the loss of *IL-6* causes cardiac dysfunction by shifting the cardiac cell populations, altering the extracellular matrix, periostin expression and disrupting critical cell-cell interactions.

Gap Junctions I (1658 – 1681)

1658/B111

Rescue of Cell Communication from Inhibition by Ouabain Is Correlated with Normalization of Sodium, but not Potassium, Levels.

M. R. Sallah, M. Ledbetter; Biology, College of the Holy Cross, Worcester, MA

Cultured MDCK cells are typically able to communicate by exchanging molecules smaller than 1000 Daltons through gap junctions composed of Connexin43. If exposed to the cardiac glycoside ouabain, an inhibitor of the Na^+, K^+-ATPase, communication is lost. Because the time course of the loss parallels the progressive loss of cell K^+ (as traced by $^{86}Rb^+$), we proposed that this correlation reflected an influence of changed ion homeostasis on the function of the gap junctions. Previous work showed that communication could be rescued by addition of amiloride (at concentrations known to block Na^+ channels) to the cultures along with ouabain. Likewise the mineralocorticoid aldosterone (known to stimulate activity of existing Na^+, K^+-ATPase pumps and induce kidney cells to synthesize new ones) protected cells from ouabain inhibition. No change in Ca^{2+} levels is associated with the loss in communication. Here we report that $^{86}Rb^+$, and hence K^+, levels are not restored in cells treated simultaneously with ouabain and amiloride or ouabain and aldosterone. To measure Na^+ levels, we used the indicator dye CoroNa Green (Invitrogen). Cells incubated with the cell-permeable acetoxymethyl ester form of the dye can take it up from the medium and trap it within by esterase hydrolysis of the acetoxymethyl groups. The trapped dye then detects intracellular Na^+, fluorescing more intensely when bound than when not bound. To correct for irregularities of cell number or loading efficiency, we included a second, Na^+-insensitive dye, calcein red-orange in the loading buffer, allowing us to use ratiometric methods to estimate Na^+ levels. We found that, as expected, ouabain treatment (1 µM for 3 hours) causes elevation of cell Na^+, but simultaneous addition of either amiloride or aldosterone substantially reduces that elevation. These data are consistent with the hypothesis that elevation of Na^+ is an early signal along the pathway of altered junctional communication. We are grateful to the National Science Foundation (DUE 0308559), the Simeon Fortin Family Foundation, and the Holy Cross Summer Undergraduate Research Program for support of this work.

1659/B112

Phosphorylation of Connexin43 at Ser373 Regulates Targeting of Gap Junctions.

G. G. Hesketh[1], J. E. Van Eyk[2,1], G. F. Tomaselli[2]; [1]Biological Chemistry, Johns Hopkins University, Baltimore, MD, [2]Medicine, Johns Hopkins University, Baltimore, MD

Gap junctions (GJs) allow direct exchange of small molecules and ions between the cytoplasm of adjacent cells. In the heart, GJs electrically couple cardiomyocytes and are necessary for synchronous electrical activation and contraction of heart muscle. Improper cardiac GJ formation, which can occur with heart disease, results in impaired electrical conduction and may result in lethal cardiac arrhythmias. Understanding the mechanisms by which GJs are formed may offer insight into the pathogenesis of cardiac disease and reveal novel therapeutic targets. Connexin43 (Cx43) is the primary GJ forming protein in the heart, and is phosphorylated extensively through its life cycle. Using mass spectrometry we identified phosphorylation of cardiac Cx43 at Ser373 in a form of the protein associated with an early phase of its life cycle. Mutation of Ser373 to alanine (S373A) and expression in HeLa cells resulted in a >90% reduction in the number of GJs formed compared with wild type (WT) Cx43 ($P<0.0001$). Confocal analysis of HeLa cells co-expressing a CD4-GFP fusion protein (which targets non-specifically to the plasma membrane) revealed that S373A localized non-specifically to the plasma membrane, rather than at cell-cell junctions to form GJs. We over-expressed both WT and S373A in cultured neonatal rat ventricular myocytes (NRMVs) using a lentiviral expression system. By western blot, S373A expressing NRVMs expressed lower levels of Cx43 associated with later phases of its life cycle compared with WT. By immuno-fluorescence, S373A expressing NRVMs had significantly reduced levels of Cx43 localized to myocyte cell-cell junctions compared with WT. We conclude that Cx43 is phosphorylated at Ser373 at an early phase in its life cycle, and that phosphorylation at Ser373 regulates the targeting of Cx43 to form GJs. The mechanism by which this likely occurs is through a phospho-Ser373 dependent protein-protein interaction required for trafficking of Cx43 to cell-cell junctions.

1660/B113

Assembly of Connexin43 into Gap Junctions Is Differentially Regulated by E-cadherin and N-cadherin.

P. Mehta; Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE

Loss of E-cadherin expression triggers dissemination of tumor cells, whereas gain of N-cadherin expression triggers motility. Hence, we investigated whether these cadherins affect the assembly of gap junctions differently. The assembly of connexin (Cx)43 into gap junctions was investigated in iso-genic clones, derived from rat liver clone9 (RL-CL9) cells, that express either E-cadherin or N-cadherin. We hypothesized that E-cadherin will facilitate the assembly whereas N-cadherin would disrupt it. The assembly of Cx43 into gap junctions was examined immunocytochemically and biochemically by detergent-solubility assay. In E-cadherin expressing clones, Cx43 was assembled into large, detergent-insoluble gap junctions whereas in N-cadherin expressing clones it remained intracellular as discrete puncta, which remained detergent-insoluble. Forced expression of N-cadherin in E-cadherin expressing clones disassembled gap junctions whereas shRNA-mediated knock down of N-cadherin induced gap junction assembly. Cell surface biotinylation revealed that Cx43 trafficked normally, and degraded with a similar kinetics, both in E-cadherin and N-cadherin clones, suggesting that the intracellular accumulation was not caused by impaired trafficking but by endocytosis prior to gap junction assembly. To test this notion, we investigated the pathway by which Cx43 was endocytosed. We used chlorpromazine to inhibit clathrin-mediated pathway and filipin and methyl-β-cyclodextrin (MβCD) to inhibit lipid raft- mediated pathway. Both filipin and MβCD increased gap junction assembly profoundly in N-cadherin expressing clones but had no effect on the assembly in E-cadherin clones. Immunocytochemical analyses revealed that Cx43 in N-cadherin expressing clones did not co-localize with early endocytic markers such as EEA1, clathrin and caveolin-1. These data suggest that N-cadherin disrupts gap junction assembly by inducing the endocytosis of Cx43 into detergent-resistant vesicles prior to its assembly into gap junctions. The diametrically opposed effect of E-cadherin and N-cadherin on gap junction assembly may rationally explain the intriguing and contrasting roles of these cadherins in regulating tumor cell motility.

1661/B114

Cx43 Phosphorylation on S330 Regulates Gap Junction Function.

J. Solan, P. Lampe; PHS, FHCRC, Seattle, WA

Assembly and maintenance of gap junctions and gap junction communication can be a highly regulated process. We have previously shown that different regions of the C-terminus of Cx43 are important in regulating these processes. We know that phosphorylation on residues S325/328/330 is important in regulating gap junction assembly and that phosphorylation on S365 and S368 can regulate channel function. In addition, we have shown that prolines 375 and 377 are part of an epitope that is found predominantly in gap junction plaques and is recognized by the monoclonal antibody IF1. Though these regions of the C-terminus are more than 35 amino acids apart in primary sequence we have found that the status of the single amino acid, S330, can regulate channel function, likely via effects on these downstream residues. Specifically, we have generated MDCK cells expressing a mutant Cx43 where S330 has been converted to alanine and found staining resembling assembled gap junctions but reduced gap junction communication compared to cells expressing wild type Cx43. However, the apparent gap junctional staining in these plaques was not recognized by the monoclonal antibody, IF1, which strongly labels gap junctions in wild type cells. The IF1 epitope includes residues that are also critical for ZO-1 binding, indicating that phosphorylation on S330 could regulate protein interactions at these distant residues. We have also found that, when examined with phosphospecific antibodies in western blot, the S330A mutant is not phosphorylated on S365. Taken together, these data indicate that phosphorylation on S330 can induce changes in conformation and/or protein interactions around residues 365-377 that may be required to create functional gap junction plaques.

1662/B115

The Role of Connexin43 in Human Gliomas.

Q. Aftab, W. Sin, C. C. Naus; Cellular and Physiological Sciences, University of British Columbia, Vancouver, BC, Canada

The malignancy of glioma tumors is correlated with lowered levels of the gap junction protein connexin 43 (Cx43). Our lab has previously shown Cx43 over-expression to be growth suppressive in C6 glioma cells, a rat model system. In addition, we also showed that the carboxy-terminal tail of Cx43 is important for C6 glioma migration and this in turn may be important for their invasiveness. We hypothesize that Cx43 suppresses tumorigenicity independently of the gap junction channel. To test this, we have begun to screen a panel of human glioma cell lines for expression and subcellular localization of Cx43. In addition, these tumor cells are being tested for intercellular coupling by dye transfer after preloading, and a number of oncogenicity parameters including proliferation rate and cell motility in wound healing and transwell migration assays. Cx43 mutants that are either defective in channel formation, hemichannel function, and adhesive properties, or

lacking different regions of the carboxy terminal tail, will be used to assess which functional domain of Cx43 is critical for its tumor suppressive functions. Our preliminary data shows growth suppression in LN229 human glioma cells upon over-expression of Cx43. The growth suppression is decreased when Cx43 with a truncated carboxy terminal tail is expressed. This suggests that the C-terminal tail of Cx43 is an important domain, and we will further explore CCN3, a negative growth regulator that we have previously identified to associate with the carboxy terminal tail of Cx43, in Cx43-mediated growth suppression in gliomas. Our study will give us insight about how Cx43 regulates growth suppression in tumor cells. This project is supported by a grant from the Canadian Institutes of Health Research.

1663/B116

Assembly of Connexin43 and Connexin32 Is Regulated Differentially in a Human Pancreatic Cancer Cell Line, BxPC3.

Kristen E. Johnson, Linda Kelsey, and Parmender P. Mehta Department of Biochemistry and Molecular Biology University of Nebraska Medical Center, Omaha, NE, 68198.

The trafficking and assembly of connexins (Cxs) into gap junctions are defective in different cancers, however, the molecular basis of this defect has not yet been defined. In particular, the role of Cxs in the pathogenesis of pancreatic cancer has not yet been explored. As a first step towards achieving this end, we have examined the expression and localization of Cx43, Cx26, Cx32 and Cx36 — the four Cxs expressed in the normal pancreas, in various pancreatic cancer cell lines. In one model cell line, BxPC3, we found that Cx43 was inefficiently assembled into gap junctions despite adequate expression and remained intracellular. In contrast, exogenously-introduced Cx32 was assembled efficiently into gap junctions in BxPC3 cells. Immunocytochemical analyses revealed that Cx43 co-localized partially with both early secretory and with endocytic markers such as GM-130, EEA1, clathrin, caveolin-1 and Lamp-1. Cell surface biotinylation revealed that Cx43 trafficked normally to the cell surface, suggesting that it was endocytosed prior to its assembly into gap junctions. To determine the pathway by which Cx43 was endocytosed, we used chlorpromazine, which inhibits the clathrin-mediated pathway, and filipin and methyl-β-cyclodextrin (MβCD), which inhibit the lipid raft-mediated pathway. We found that both classes of inhibitors attenuated the endocytosis of Cx43 and facilitated its assembly into gap junctions. Our findings suggest that the assembly of Cx43 and Cx32 into gap junctions upon arrival at the cell surface is subject to Cx-specific distinct regulatory mechanisms at the site of cell-cell contact.

1664/B117

Inhibition of Cx43 Hemichannels Prevents Cortical Astrocytes Death Induced by Hypoxia in High Glucose Concentration.

J. A. Orellana[1], D. E. Hernandez[1], P. Ezan[3], V. Velarde[1], M. Bennett[2], C. Giaume[3], J. Saez[1,2]; [1]Departamento de Fisiología, Pontificia Universidad Católica de Chile, Santiago, Chile, [2]Dept. Neuroscience, Albert Einstein College of Medicine, Bronx, NY, [3]INSERM U840, Collège de France, Paris, France

Hyperglycemia during brain ischemia increases the extent of injury and causes massive neuronal and astroglial death. In vitro ischemia models have shown increased opening of astroglial connexin hemichannels (HCs) and decreased coupling by gap junctions (GJC). However, the effects of high glucose concentrations during hypoxia on astrocytic Cx43 channels remain unknown. We evaluated if high glucose levels during hypoxia influence HCs and GJC during reoxygenation and whether these changes promote astroglial death. Cortical astrocytes cultured from rats or from Cx43-/- and wild type mice were subjected to reoxygenation after 3 or 6h of hypoxia with different glucose concentrations. HC activity was evaluated by ethidium uptake and GJC by intercellular dye transfer measurements. Astroglial death was evaluated by uptake of rhodamine-dextran (10kDa). After 3h of hypoxia in high glucose (12-37mM) dye uptake increased and dye coupling decreased transiently in a concentration dependent manner reaching maximal response with 27mM glucose. Six hours of hypoxia in 27mM glucose the highest increased dye uptake increase (to 400±23% of control at ~1h reoxygenation) and dye coupling reduction (to 10±2% of control at ~1h reoxygenation remained constant over 3.5h of reoxygenation and were not observed in Cx43-/- astrocytes. Moreover, 6 h hypoxia in 27mM glucose induced 43±4 % astroglial death after 6h of reoxygenation in wild type but not in Cx43-/- astrocytes. Astroglial death was prevented by Cx43 mimetic peptides (GAP26, GAP27) that blocked HCs and did not affect GJC. Thus, HCs may be a therapeutic target to reduce cell death following stroke accompanied by hyperglycemia.

1665/B118

Microtubules and Actin Differentially Influence Remodeling of Connexin43 Gap Junctions.

A. Hunter, R. Gourdie; Department of Cell Biology and Anatomy, Medical University of South Carolina, Charleston, SC

Regulation of Cx43 gap junction (GJ) size and organization is poorly understood. Previously we showed that fusion of GFP to the C-terminus of Cx43 leads to the formation of aberrantly large GJs. Cx43 GJs are resistant to Triton detergent extraction, yet Cx43-GFP GJs are largely Triton-soluble. Interestingly, Triton-insoluble Cx43-GFP localizes predominately to plaque edges—the site of GJ growth—suggesting that GJ edges are stabilized by cytoskeletal interactions that influence GJ size. Fluorescence labeling revealed minimal interaction of actin filaments with Triton-insoluble Cx43-GFP. In contrast, plaques composed of native Cx43 were extensively colocalized with actin polymer. However, Cx43-GFP plaques appear to acquire more microtubule contacts than native Cx43 GJs. Live cell imaging showed GJs containing a mix of Cx43-GFP and native Cx43 are more dynamic than plaques comprised solely of Cx43-GFP. Inhibition of either actin polymerization or Cx43 interaction with the actin binding protein ZO-1 suppressed the dynamics of mixed Cx43 GJs. These results suggest that Cx43 C-terminal elements, including the PDZ binding domain, determine cytoskeletal interactions at GJ edges, with ZO-1-mediated actin connections promoting active GJ remodeling, whereas microtubule contacts confer GJ stability and growth. Supported by NIH grants HL07260, K12GM081265, HL56728, HL082802.

1666/B119

Selectivity of Gap Junctions Composed of Cx43-S365A, -S365D and -S368A: Influence of PKC Activation.

J. F. Ek-Vitorin, J. M. Burt; Department of Physiology, University of Arizona, Tucson, AZ

Transjunctional diffusion of endogenous molecules supports the functional coordination of tissues and organs. Restraining the extent and specificity of this diffusion may be equally important for such coordination, particularly during physiologic challenges. During such challenges, dynamic connexin-specific changes in selectivity could fulfill present, specific and relevant cellular needs. PKC-induced

phosphorylation of Cx43 at S368 (pS368) alters junctional permselectivity (P_j/g_j), but analogous changes in dye selectivity have not been reported. Recent data indicate that pS368 is alternative to, and inhibited by, phosphorylation at S365 (pS365), so that pS368 occurs in the absence of pS365. To explore possible consequences of pS365:pS368 balance on junctional selectivity, we performed dual dye injections with measurement of transjunctional diffusion rates (P_{dye}) in cell pairs expressing the Cx43 mutants S368A, S365A and S365D. We hypothesize that S368A and S365D would similarly impair pS368, while S365A would be permissive to pS368. To assess size and charge selectivity, Alexa350 (Stokes radius 4.3Å, charge 1-) was co-injected with Alexa488 (5.8Å, 2-) or NBD-M-TMA (4.4Å, 1+), respectively. Charge and size selectivities of Cx43wt and Cx43-S368A are similar and not modified by PDGF treatment (in agreement with previous findings with TPA). Untreated Cx43-S365A (n=60) junctions showed no charge selectivity ($P_{Alexa350}/P_{NBD\text{-}M\text{-}TMA}$ ~1) in ~80% of the pairs explored; the remaining pairs displayed anionic selectivity (mean ~7.7). In PDGF-pretreated Cx43-S365A (n=14), no significant selectivity was detected ($P_{Alexa350}/P_{NBD\text{-}M\text{-}TMA}$ ~1.1), while junctions showed higher P_{dye} values than the control group. In stark contrast, TPA-treated Cx43-S365A (n=8) pairs displayed anionic selectivity ($P_{Alexa350}/P_{NBD\text{-}M\text{-}TMA}$ ~8), although P_{dye} values were greatly decreased compared to the control pairs. Experiments with Cx43-S365D cells are under way. The present data suggest that Cx43 phosphorylation regulates the selectivity of gap junctions, but overall high P_j might conceal that selectivity. Supported by: HL058732.

1667/B120

Cell-Type Specific Connexin 43 Phosphorylation in Response to In Vivo Application of Oxidized Phospholipids.

S. R. Johnstone[1,2], M. J. Rizzo[1,2], B. Isakson[1,2]; [1]Robert M. Berne Cardiovascular Research Center, University of Virginia, Charlottesville, VA, [2]Department of Molecular Physiology and Biological Physics, University of Virginia, Charlottesville, VA

During atherogenesis modification of vascular cell connexin (Cx) expression occurs, particularly for the Cx43 isoform. Moreover, post-translational phosphorylation of sites in the carboxyl tail of Cx43 has been associated with alterations in vascular cell migration and proliferation. However, it is not known what causes these changes. In order to identify Cx43 changes that are important for atherogenesis, we compared the total (Cx43-T) protein expression and serine phosphorylation (pS) at S279/282 and S368 in carotids isolated from wild type mice fed standard and high fat diets. While increases in dietary fat did not alter Cx43-T, significantly elevated levels of Cx43 pS279/282 and pS368 in vascular smooth muscle cell (VSMC) layers, but only pS368 in endothelial cells (EC) were observed. Based on these observations we investigated the in vivo effects application of the specific oxidized phospholipid (OxPL) species found during atherogenesis, 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphorylcholine (PGPC) and 1-palmitoyl-2-oxovaleroyl-sn-glycero-3-phosphorylcholine (POVPC) on alterations in Cx43-T, pS279/282 and pS368. Application of both lipids induced differential phosphorylation of Cx43 in VSMC: PGPC predominantly affected pS368, whereas POVPC mainly affected pS279/282 and was also shown to reduce Cx43-T. The POVPC-induced pS279/282 correlated with an increase in the number of VSMC. We confirmed the cell type specific effects by comparisons of wild type versus conditional vascular EC (EC-/-) and VSMC (VSMC-/-) Cx43 knockout mice. We conclude that a high fat diet promotes a cell-type specific increase in Cx43 phosphorylation, which is mediated by specific phospholipid oxidation products. These Cx43 phosphorylation events may be crucial to promoting VSMC proliferation during atherogenesis.

1668/B121

Connexin43 Protein Expression Influences the Rate of Cell Migration in Wound Healing Assays.

A. Mendoza-Naranjo, K. T. Power, P. Cormie, C. Wang, D. L. Becker; Cell & Developmental Biology, University College London, London, United Kingdom

Active downregulation of the gap junction protein connexin 43 (Cx43) accelerates skin wound repair by influencing several processes including cell migration. If Cx43 protein is not down regulated, as is seen in models diabetic wound healing, migration is compromised. However, the mechanisms behind accelerated migration and compromised migration remain elusive. This work focused on dissecting out the mechanisms underlying the influence of Cx43 on cell migration. Using time-lapse microscopy we have investigated the rates of migration of 3T3 fibroblasts in a scrape wound healing assay after transfection with either empty pIRES-GFP vectors containing wild type Cx43 or dominant negative Cx43. Migration, adhesion and cytoskeletal dynamics were also assessed in fibroblasts with Cx43 levels elevated pharmacologically or reduced by transfection with pSuper-Cx43RNAi. We further demonstrated, using both in vivo and in vitro models, that the accelerated movement induced after silencing Cx43 in fibroblasts correlates with reduced cell adhesion, cytoskeletal changes and altered expression/distribution of adhesion proteins at the wound edge. These findings may have therapeutic implications for the management of wound healing and the development of ways to manipulate the cells at the injury site in order to encourage them to crawl forward and close the wound more rapidly.

1669/B122

Phosphatidylinositol 3-Kinase/Protein Kinase Akt Regulates Connexin43 Expression through mRNA Stability in Osteoblasts.

R. Bhattacharjee[1,2], M. Kaneda[1], K. NAKAHAMA[1], I. Morita[1,2]; [1]Cellular Physiological Chemistry, Tokyo Medical and Dental University, Tokyo, Japan, [2]Global Center of Excellence program, International Research Center for Molecular Science in Tooth and Bone Disease, Tokyo Medical and Dental University, Tokyo, Japan

OBJECTIVE: Cells of the osteoblastic lineage play a critical role in bone development and remodeling. Gap junction intercellular communication is a characteristic feature of osteoblasts and may be essential for their normal functioning. Osteoblastic cells express Connexin (Cx) 43 as the major gap junction protein. The level of cell-to-cell communication can be actively adjusted by multiple mechanisms including changes in Cx expression. On the other hand, PI3K/Akt is one of the key players in the signaling of potent bone anabolic factors. This pathway has been shown to regulate osteoblast's activity by promoting their differentiation and survival. The aim of this study was to test the hypothesis that PI3K/Akt signaling affects Cx43 expression in MC3T3-E1 osteoblastic cells. METHODS: Western blot, Real time PCR was used for the quantification of protein and gene expression respectively. FRAP (Fluorescence Recovery After Photobleaching) method was used to measure gap junction intercellular communication. Promoter activity of Cx43 was examined by Dual-luc promoter assay and the mRNA stability was studied by actinomycin D chase experiment. RESULTS: We found that LY294002, a specific PI3K inhibitor, significantly decreased Cx43 mRNA and protein levels. Furthermore, a dominant-negative Akt expression reduced

both Cx43 expression and gap junction activity. Interestingly, Cx43 promoter assay showed that the transcription was not affected by LY294002. On the other hand, actinomycin D chase experiment revealed that the pharmacological inhibition of PI3K/Akt or the forced expression of dominant-negative Akt downregulated Cx43 expression post-transcriptionally via mRNA instability. CONCLUSION: Our results suggest that PI3K/Akt has an important role in the regulation of Cx43 expression through Cx43 mRNA stability.

1670/B123

Teratogenicity/Embryotoxicity of Valproic Acid (VPA) Influences Connexin43 (Cx43) Expression in the Embryonic Chick Heart Micromass (MM) Model System. Ahir B K and Pratten M K., School of Biomedical Sciences, Queen's Medical Centre, University of Nottingham, Nottingham, UK.

B. Ahir; School of Biomedical Sciences, The University of Nottingham, Nottingham, United Kingdom

Congenital abnormalities of heart are the leading cause of infant mortality. Maternal exposures to valproic acid (VPA), one of the most widely consumed antiepileptic drugs during pregnancy in the world, has been associated with embryological malformations and developmental defects. The exact underlying mechanisms of teratogenesis are unclear. Cardiac muscle cells are coupled by gap junctions, clusters of low-resistance multisubunit transmembrane channels composed of connexins (Cx). There are three major connexins, Cx40, Cx43 and Cx45. The expression of one of these three major connexins, Cx43 present in cardiomyocytes, is known to be developmentally regulated. The study reported here investigates the idea that alterations in gap junction proteins are one of the mechanisms by which teratogens may act. Specifically, that molecule known to be teratogenic in the human may, by this mechanism, cause alterations in cardiomyocyte differentiation resulting in abnormal heart development. Thus, the possible effect of VPA on gap junction protein (Cx43) formation was studied in chick cardiomyocytes in micromass culture. White Leghorn 5-day old chick embryo hearts were dissected to produce a cardiomyocyte cell suspension in DMEM culture medium. Cell cultures were incubated at 370C in 5% CO2 (V/V) and observations were made for cellular differentiation (cell beating) at 24 hours, 48 hours and 144 hours. Cell culture activity was assessed using the Resazurin reduction assay for determining the metabolic activity of cultures and total protein determined via the Kenacid blue assay. Effects on Cx43 expression was analysed and quantified using cytofluormetery and immunocytochemisrtry. It was found that VPA exposure up to (100-2000µM) induced no cytotoxicity in the heart MM model system, whilst all VPA concentrations tested significantly reduced cell beating ($P<0.01$), and that this coincided with and decrease in the expression of Cx43. The results confirmed that Cx43 is affected by the potential human teratogenic/embryotoxic effects of VPA test molecule on embryonic chick heart micromass model which provides useful in vitro embryotoxicity testing for mechanistic studies of perturbation of embryonic heart development.

1671/B124

Decreased Levels of Cx43 Result in Ameloblast Dysregulation and Enamel Hypoplasia in Gjajrt/+ Mice.

K. Toth, Q. C. Shao, D. W. Laird; Anatomy & Cell Biology, University of Western Ontario, London, ON, Canada

Coordinated differentiation of the ameloblast cell layer is essential to enamel matrix protein deposition and subsequent mineralization. It has been hypothesized that his process is governed by Cx43-based gap junctional intercellular communication as oculodentodigital dysplasia (ODDD) patients harboring autosomal-dominant mutations in Cx43 exhibit enamel defects typically resulting in early adulthood tooth loss. To assess the role of Cx43 in tooth development we employ a mouse model of ODDD that harbors a G60S mutant, $Gja^{Jrt}/+$, and appears to exhibit tooth abnormalities that mimic the human disease. We found that Cx43 plaques at all stages of ameloblast differentiation, as well as within the stratum intermedium, were reduced by >50% in $Gja^{Jrt}/+$ incisors compared to wild-type littermate controls. To characterize the $Gja^{Jrt}/+$ mouse tooth phenotype resulting from reduced Cx43 gap junctions during development, mice were sacrificed at tooth eruption (postnatal day 7) and two weeks later (postnatal day 21). Histological and immunohistochemical analysis of longitudinal sections through the lower incisors were used to assess tooth development. A severely disorganized $Gja^{Jrt}/+$ ameloblast cell layer and retention of proteinaceous material were observed at stages when the cells were active in secretion and mineralization. Differences in enamel thickness only become apparent after tooth eruption and incisor exposure to the oral cavity suggesting that enamel integrity is compromised, leading to rapid erosion. Additional analysis of incisors from 21 day old mutant mice revealed that they were longer with a thicker dentin layer than their wild-type littermates, which may reflect a mechanical stress response to the depleted enamel layer. Together, these data show that reduced levels of Cx43 gap junctions result in ameloblast dysregulation, enamel hypoplasia and secondary tissue responses. Future studies hope to elucidate the molecular mechanism behind these defects. Supported by CIHR to DWL.

1672/B125

Aberrant Cx43 Localization and Function in Cells and Tissues Obtained from Oculodentodigital Dysplasia Patients Mimic Findings from Mutant Mouse Models of Cx43-Linked Disease.

Q. C. Shao[1], J. Churko[1], J. Sampson[2], K. Flanigan[2], K. Swoboda[2], D. Laird[1]; [1]Anatomy & Cell Biology, The University of Western Ontario, London, ON, Canada, [2]Department of Neurology, University of Utah School of Medicine, Salt Lake City, UT

Oculodentodigital dysplasia (ODDD) is primarily an autosomal dominant human disease caused by 41 mutations in the GJA1 gene encoding the gap junction protein Cx43. Although 3 mutant mouse models of ODDD have now been reported with the anticipation that they mimic the human disease, direct correlation with Cx43 localization, distribution, and functional data from ODDD patients is lacking. To that end we have generated a human skin fibroblast cell line from an ODDD patient harboring the V216L mutation. Similar to what we observed from a variety of primary cells obtained from the Gja1Jrt/+ mutant mouse model of ODDD, the ODDD patient fibroblasts expressed Cx43 with evident gap junction plaques but there was a notable reduction in the most phosphorylated species of Cx43 compared to normal human skin fibroblasts. In addition, like primary cultured fibroblast cells derived from Gja1Jrt/+ mutant mouse, the incidents of dye coupling in ODDD fibroblasts were found to be one third of that found in normal human skin fibroblast cells. In a migration assay, ODDD fibroblasts were found to migrate faster than normal human skin fibroblasts at 24hr. Skin biopsies obtained from a second ODDD patient, as well as from the unaffected parent of this patient, exhibited the presence of Cx43 in suprabasal keratinocytes but there was a remarkable reduction in the prevalence of gap junctions in the epidermis from the ODDD patient. Together these studies would suggest that information regarding Cx43 localization, phosphorylation, and function obtained from the Gja1Jrt/+ mouse model of ODDD is in fact recapitulated in human cells and

tissues from ODDD patients. In addition, this is the first human ODDD cell line generated and utilized to compare a human ODDD genotype with cellular phenotypes. Supported by the Canadian Institutes of Health to DWL.

1673/B126

The Connexin43-Interacting Protein Cip85 Regulates the Trafficking and Degradation of Connexin43.

K. Cochrane[1], J. Berestecky[2], C. Kitamura[1], A. Lau[1]; [1]Cancer Research Center of Hawaii, University of Hawai'i, Honolulu, HI, [2]Microbiology and Biotechnology, Math/Science, Kapiolani Community College/University of Hawai'i, Honolulu, HI

The gap junction protein connexin43 (Cx43) is expressed in the mammalian heart and other tissues where it plays a critical role in tissue function and homeostasis. Yeast-two hybrid screening has identified a mouse connexin43-interacting protein of 85 kDa (Cip85/SGSM3). Cip85 or its homologues are ubiquitously expressed in mouse and human tissues and are conserved in human, D. melanogaster, and C. elegans. CIP85 contains a Src homology 3 (SH3) domain known to bind to proline-rich regions of target proteins, a TBC domain found in the Tre-2, BUB2p, and Cdc16p proteins that exhibits Rab GTPase activator activity, a RUN domain that plays a role in Ras-like GTPase signaling, and a short coiled-coil protein-binding motif. The human homologue of mouse CIP85 has been shown to function as a Rab5 GAP. The interaction between CIP85 and Cx43 has previously been characterized and appears to induce the turnover of Cx43 through the lysosomal pathway. We have proposed that CIP85 may be involved in the trafficking of Cx43 from the plasma membrane. We have generated monoclonal antibodies against the full-length mouse CIP85 protein to allow for further characterization of this interaction and its functional importance. Mice were immunized with full-length CIP85 protein, purified from bacteria, and splenocytes from these mice were fused with mouse myeloma cell lines by standard hybridoma production techniques. Enzyme-linked immunosorbent assay (ELISA) and western blot analysis demonstrated antibody specificity to the conserved domains of CIP85. The antibodies also immunoprecipitated endogenous CIP85 from the lysates of multiple mammalian cell lines and detected endogenous CIP85 in mammalian cells via immunofluorescence laser-scanning confocal microscopy. Isotyping revealed that the antibodies contain kappa light chains and either immunoglobulin G1, G2a, or G2b heavy chains. We are currently using these antibodies to determine the subcellular localization of endogenous CIP85 and its involvement in the Rab-mediated trafficking of Cx43 and it's degradation by lysosomes.

1674/B127

Decreased Connexin43 and Gap Junctional Communication Alter Cell Migration.

B. DeFranco, B. M. Nickel, S. Murray; Cell Biology and Physiology, University of Pittsburgh - School of Medicine, Pittsburgh, PA

Gap junctions are specialized intercellular membrane channels that play a pivotal role in coordinating cellular migration. However, whether migrating cells require gap junction structures or function is indefinite. We previously demonstrated that gap junction plaques are retained and remain functional in cells that migrate in a sheet-like procession. We now propose that both gap junction structure and intercellular communication are required for coordinating migrating cells. We have utilized an in vitro, wound healing, model for initiating and monitoring cell movement. To assess the effect of altering intercellular communication on cell migration, we introduced, into cell cultures, mimetic peptides (Gap 26 and 27) that specifically inhibit gap junction coupling. Functional dye coupling was confirmed with the gap junction permeable dye, Lucifer yellow. To reduce the availability of gap junction plaques, siRNA, specifically targeting the mRNA of the gap junction protein connexin 43 (Cx43), were introduced into cells. Cx43 protein expression and gap junction plaque formation were confirmed with western blot and immunofluorescence, respectively. Reduced gap junction mediated dye coupling was observed in cells treated with mimetic peptides. In communication impaired cells migration was slowed, thus confirming the necessity for gap junction communication in efficient cell migration. Furthermore, cell motility was drastically hindered when Cx43 gap junction plaque structures were reduced. Our results are consistent with sustained gap junction plaques and intercellular communication for coordinated cell movement during development and maintenance of tissue architecture. Supported by NSF grants MCB-0444398 and 0647748.

1675/B128

Interferon-Gamma Inhibits Enterocyte Migration by Reversibly Displacing Connexin43 from Lipid Rafts.

C. Leaphart, S. Dai, S. Gribar, W. Richardson, J. Ozolek, J. Bruns, M. Branca, O. A. Weisz, C. Sodhi, D. Hackam; Surgery, Children's Hospital of Pittsburgh, Pittsburgh, PA

Intestinal inflammation is associated with the release of interferon gamma (IFN) by enterocytes and delayed intestinal restitution. We have recently demonstrated that IFN inhibits enterocyte migration by impairing enterocyte gap junctions, intercellular channels that are composed of connexin43 (Cx43) monomers and that are required for enterocyte migration to occur. The mechanisms by which IFN inhibits gap junctions are incompletely understood. Lipid rafts are cholesterol-sphingolipid rich microdomains of the plasma membrane that play a central role in the trafficking and signaling of various proteins. We now hypothesize that Cx43 is present on enterocyte lipid rafts, and that IFN inhibits enterocyte migration by displacing Cx43 from lipid rafts in enterocytes. We now confirm our previous observations that intestinal restitution is impaired in NEC, and demonstrate that Cx43 is present on lipid rafts in IEC-6 enterocytes. We show that lipid rafts are required for enterocyte migration, that IFN displaces Cx43 from lipid rafts, and that the phorbol ester PMA restores Cx43 to lipid rafts after treatment with IFN in a protein kinase C-dependent manner. IFN also reversibly decreased the phosphorylation of Cx43 on lipid rafts, which was restored by PMA. Strikingly, restoration of Cx43 to lipid rafts by PMA or by transfection of enterocytes with adenoviruses expressing wild-type Cx43 but not mutant Cx43 is associated with the restoration of enterocyte migration after IFN treatment. Taken together, these findings suggest an important role for lipid raft-Cx43 interactions in the regulation of enterocyte migration during exposure to IFN.

1676/B129

Connexin43.4 Is Required for Kupffer's Vesicle Formation during Zebrafish Left-Right Patterning.

J. M. Hatler[1], J. J. Essner[2], R. G. Johnson[1]; [1]Genetics, Cell Biology and Development, Univ. of Minnesota, Minneapolis, MN, [2]Genetics, Development and Cell Biology, Iowa State Univ., Ames, IA

Early patterning of vertebrate embryos involves induction of asymmetry across the left-right (L-R) axis and is critical for development of multiple organs. This patterning is driven by a highly conserved signaling cascade on the left side, thought to be asymmetrically directed and

propagated by ciliated cells within the embryonic node of mammals; a similar structure exists in zebrafish, called Kupffer's vesicle (KV). Given our detection of Cx43.4 expression in KV, we theorized that this Cx (closely related to mammalian Cx45) plays a role in establishing L-R asymmetry. Following morpholino (MO) knockdown of Cx43.4, normal, leftward looping of the heart tube was reversed in 41% of the embryos, with 50% reversal being the theoretical maximum for randomization. We have shown that injection of zebrafish Cx43.4 or human Cx45 mRNA can partially rescue reversed heart looping, indicating that Cx43.4 is specifically required for L-R asymmetry, putatively in a conserved developmental process. Characterization of L-R defects by in situ hybridization demonstrated that genes normally expressed on the left were misexpressed in Cx43.4-deficient embryos. We also showed that Cx43.4 is not required for KV cell specification or ciliogenesis, using tubulin immunofluorescence on Cx43.4-deficient embryos. In contrast, examination of KV structure using a KV-specific membrane marker revealed defects in morphogenesis, e.g., smaller and non-inflated KVs in morphants. We are examining the mechanism(s) responsible for the defects in KV development and hypothesize that Cx43.4 is required for establishing or maintaining polarized epithelial cells capable of forming an inflated, functional KV. Since connexins can function both as hemichannels (opening to the extracellular space) and as intercellular channels, we tested whether a mutant form of Cx43.4 (C64S) that has been designed to form only hemichannels, not gap junctions, could rescue the left-right defect. Preliminary studies suggest this mutant is sufficient to rescue KV lumen formation. One model addressing formation of the KV lumen highlights the role of apical Cx43.4 hemichannels and P2 receptors, which together drive fluid flow into the developing lumen.

1677/B130

Luteinizing Hormone Causes MAP Kinase-Dependent Phosphorylation and Closure of Connexin43 Gap Junctions in Mouse Ovarian Follicles: One of Two Paths to Meiotic Resumption.

R. Norris[1], M. Freudzon[1], A. E. Cowan[2], L. M. Mehlmann[1], A. M. Simon[3], D. L. Paul[4], P. D. Lampe[5], L. A. Jaffe[1]; [1]Dept. of Cell Biology, UConn Health Center, Farmington, CT, [2]Center for Cell Analysis and Modeling, UConn Health Center, Farmington, CT, [3]Dept. of Physiology, Univ. of Arizona School of Medicine, Tucson, AZ, [4]Dept. of Neurobiology, Harvard Medical School, Boston, MA, [5]Fred Hutchinson Cancer Research Center, Seattle, WA

In mammalian oocytes contained within ovarian follicles, meiotic arrest at prophase I is relieved by the action of luteinizing hormone (LH) on the surrounding somatic cells. This process is not fully understood, but one long-standing idea is that LH causes gap junctions within the follicle to close, interrupting the transfer of a meiosis-inhibitory signal to the oocyte. While several observations support this hypothesis, it has not been directly tested. To investigate this idea, we microinjected fluorescent tracers into live antral follicle-enclosed mouse oocytes, and demonstrate for the first time that LH causes a decrease in the gap junction permeability between the somatic cells, prior to nuclear envelope breakdown (NEBD) in the oocyte. The decreased permeability results from MAPK-dependent phosphorylation of connexin 43 on serines 255, 262, and 279/282. We then tested whether inhibition of gap junction communication is sufficient and necessary for the reinitiation of meiosis. Inhibitors that reduced gap junction permeability caused NEBD, but an inhibitor of MAPK activation that blocked gap junction closure in response to LH did not prevent NEBD. Thus both MAPK-dependent gap junction closure, and another pathway that is independent of the junction closure, function in parallel to ensure that meiosis resumes in response to LH.

1678/B131

Proteasomal Degradation of Connexin43 Facilitated by CIP75.

V. Su[1], A. Knutson[1], K. Lau[1], W. Kurata[1], J. Berestecky[2], A. Lau[1]; [1]Natural Products and Cancer Biology, Cancer Research Center of Hawaii, Honolulu, HI, [2]Microbiology and Biotechnology, Kapiolani Community College/University of Hawaii, Honolulu, HI

Control of connexin43 (Cx43) protein levels can have an important role in regulating cell communication. Cx43 is known to be rapidly degraded with a half-life of 1.5-5 hours. While it has been shown that Cx43 degradation occurs via the lysosomal and proteasomal pathways, how this happens and what controls the degradation is not clear. We have previously identified a novel Cx43-interacting protein, CIP75. CIP75 is a member of the UbL (ubiquitin-like domain)-UBA (ubiquitin-associated domain) family of proteins which have been implicated in proteasomal degradation. CIP75 is involved in Cx43 turnover, possibly through degradation via the proteasomal pathway. We have previously shown that CIP75 can interact with both Cx43 and proteasomal components, specifically subunits of the 19S cap. CIP75 also stimulates Cx43 degradation, via the proteasome. We further investigate the role of CIP75 acting in the Cx43 proteasomal degradation pathway, the possible role of ubiquitination of Cx43, as well as whether CIP75 may potentially have a more general role in the proteasomal degradation pathway. To aid our studies of the role of CIP75, we have developed monoclonal antibodies and have characterized a variety of clones that have specificities to the N-terminal UbL domain, the C-terminal UBA domain, or the middle section of the protein. Initial analysis of specificity was done through ELISA and Western blot assays. The monoclonal antibodies were further characterized for the ability to immunoprecipitate endogenous CIP75 in various cell lines, as well as to detect CIP75 through immunofluorescence where the majority of antibodies were able to recognize CIP75 through immunoprecipitation and immunofluorescence assays. Thus, we have generated and characterized the specificity and functionality of monoclonal antibodies to CIP75, a tool that will be invaluable for future studies of CIP75.

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Androgen Suppression of the Gap Junction Protein Connexin43 in the Rat Caput Epididymis Involves c-Src, but Not PKCcat or TJP1.

S. Edwards, D. Cyr; INRS-Institut Armand-Frappier, Laval, QC, Canada

Intercellular communication in the epididymis is mediated by gap junctions (GJ). Our laboratory has previously shown that the intracellular targeting of Cx43 in the initial segment (IS) of the epididymis is mediated by testicular androgens. This offers a unique mechanism to study the regulation of Cx43 targeting. The objectives of the present study were to: 1) characterize the localization and expression of Cx43 regulators cellular Rous sarcoma virus (c-Src) and cyclic AMP-dependent protein kinase A catalytic subunit (PKAcat) in the rat epididymis, 2) determine the segment specific androgen regulation of Cx43 and its potential regulators (c-Src, PKAcat and tight junction protein 1 (TJP1)) by orchidectomy (orch) and androgen replacement, and 3) determine if there is a direct, androgen-dependent association between Cx43 and/or c-Src, PKCat and TJP1 as determined by immunoprecipitation. PKAcat and c-Src were expressed in all segments of the

epididymis and localized between basal and principal cells where Cx43 has been previously reported. Orch resulted in an apical accumulation of c-Src, which was reversed by testosterone (T) replacement. The cellular targeting of PKAcat was unaltered by either orch or T replacement. In the IS and caput (CT) regions of the epididymis, orch decreased the expression of c-Src, PKAcat and TJP1. T-replacement partly reversed this decrease. Unphosphorylated Cx43 expression was unchanged by orch in the IS, whereas the P2 phosphorylated state was decreased. In contrast, Cx43 expression was significantly increased in the CT of orch rats, an effect that was reversed by T replacement, whereas the P2 phosphorylated state was unchanged. A direct association between Cx43 and its regulators was established in the CT and was T-dependent for c-Src. Our results indicate that Cx43 expression is inhibited by T in the CT and that this may be mediated by a direct association with c-Src. Overall these results suggest that c-Src may play an important role in regulating Cx43 targeting in the epididymis. Supported by Fondation Armand-Frappier, FQRNT and NSERC.

1680/B133

Histone Deacetylase Inhibition Reduces Ventricular Gap Junction Coupling.

X. Lin[1], L. Coleman[1], J. Gemel[3], A. Glass[2], S. M. Taffet[2], E. Beyer[3], R. D. Veenstra[1]; [1]Pharmacology, SUNY Upstate Medical University, Syracuse, NY, [2]Microbiology and Immunology, SUNY Upstate Medical University, Syracuse, NY, [3]Pediatrics, University of Chicago, Chicago, IL

Histone deacetylase (HDAC) inhibitors have shown great potential as pharmaceutical therapies for various forms of cancer, cardioprotection, and neuroprotection. However, adverse cardiac arrhythmias associated with these agents have been reported. The mechanisms for the cardiotoxicity are not clear, though reduced intercellular coupling could contribute to reported ECG aberrances. Relatively little is known about the biological effects of HDAC inhibitors on cardiac gap junctions. In this study, we investigated the effects of trichostatin A (TSA), the protoypical nonselective HDAC inhibitor, on cardiac gap juctions in cultured neonatal mouse ventricular myocytes. Fluorimetric HDAC activity assays indicate that ventricular myocytes are nearly 10-fold more sensitive to TSA inhibition than HeLa cells. Relative HDAC gene expression levels, determined by real-time PCR analysis, were HDACs 2/4/5 > 6/7/9/10/11 > 3/8 > 11. TSA treatment (>12 h) led to a dose-dependent (10-200 nM) decrease in Cx43 protein levels as detected by immunoblotting. Quantitative confocal image analysis showed that treatment with 100 nM TSA decreased Cx43 gap junction plaque area by about 75%. Dual whole cell patch clamp experiments demonstrated that treatment with 100nM TSA (12 h) decreased the total cardiac gap junction conductance (gj) by 40%. TSA treatment slowed transjunctional voltage (Vj)-dependent inactivation and reduced the maximum gj (facilitation) during the recovery phase in a dose-dependent manner (10-200 nM). TSA treatment also reduced the cardiac gap junction unitary channel conductance (γj) to 30-60 pS with an average slope γj of 50 pS. We conclude that TSA regulates cardiac gap junctions at every level, from connexin expression and gap junction assembly (N) to gating properties (Popen) and channel conductance (γj). These results imply a previously unrecognized mechansim of regulation of cardiac gap junctions: through modulation of protein acetylation/deacetylation. Which HDACs contribute to these different modulatory gap junction effects and whether the effects result from Cx43 or accessory protein acetylation remain to be determined.

1681/B134

A Model of the Gap-Junctional Hemichannel (GJH) Transmembrane Pore Based on Spectroscopic Distance Measurements.

S. C. Lee[1], C. F. Hamilton[1], S. H. Choi[1], L. Reuss[1,2], G. A. Altenberg[1,2]; [1]Cell Physiology and Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, TX, [2]Center for Membrane Protein Research, Texas Tech University Health Sciences Center, Lubbock, TX

Gap-junctional channels are formed by head-to-head docking of GJH, connexin hexamers. The GJH models based on low-resolution 2D diffraction data show 4 transmembrane α-helical structures from each connexin monomer, but do not provide sufficient detail for the assignment of the helices. The transmembrane helices (M1 to M4) have been named A, B, C and D, with helices B and C lining the pore. Here, we calculated distances between selected homologous residues (same residue in different monomers) in helices of diametrically-opposed monomers. Purified GJH reconstituted into liposomes were formed by functional single-cysteine Cx43 mutants, with one donor (chelated Tb3+)-labeled and one or more fluorescent acceptor-labeled monomers. Luminescence resonance energy transfer (LRET) was employed to calculate distances between donor-acceptor probes, from the sensitized acceptor emission lifetimes. The calculated distances from 8 positions per helix (4 on the cytoplasmic and 4 on the extracellular side) allowed for the assignment of M2 (~45 Å) and M3 (~41 Å) as pore-lining helices, and M1 (~57 Å) and M4 (~60 Å) as peripheral helices. On the extracellular side (narrower side of the pore), the distances were ~23 and ~40 Å, for M2 and M3 residues, respectively. These data, including the evidence for tilting of M2 (deduced from a shortening of distance of ~20 Å near the extracellular side of the GJH compared to the cytoplasmic side), identify M2 as helix C, the primary pore-lining helix. Similar experiments allowed for the assignment of M1 as helix A and M4 as helix D. The excellent agreement between the LRET calculated distances and those of the available low-resolution structures validate the LRET measurements and strongly suggest that the conformation of the GJH in the 2-D crystals is similar to that of the functional GJH in the lipid bilayer. This work was supported in part by NIH grants DC007150, GM068586 and GM79629, and American Heart Association, Texas Affiliate grant 0755002Y.

Membrane Channels (1682 – 1697)

1682/B136

Functional Stoichiometry of the Unitary Calcium-Release-Activated Calcium Channel Revealed by Single Molecule Imaging.

W. Ji[1], P. Xu[1], Z. Li[1], J. Lu[1], L. Liu[2], Y. Zhan[2], B. Hille[3], T. Xu[1,2], L. Chen[1]; [1]Institute of Biophysics, Beijing, China, [2]Institute of Biophysics and Biochemistry, Huazhong University of Science and technology, Wuhan, China, [3]Department of Physiology and Biophysics, University of Washington School of Medicine, Seattle, WA

Two proteins, STIM1 in the endoplasmic reticulum and Orai1 in the plasma membrane are required for the activation of calcium release-activated calcium (CRAC) channels at the cell surface. How these proteins interact to assemble functional CRAC channels has remained uncertain. Here we determine how many Orai1 and STIM1 molecules are required to form a functional CRAC channel. We engineered

several genetically expressed fluorescent Orai1 tandem multimers and a fluorescent, constitutively active STIM1 mutant. The tandem multimers assembled into CRAC channels as seen by rectifying inward currents and by cytoplasmic calcium elevations. CRAC channels were visualized as fluorescent puncta in total internal reflection microscopy. With single-molecule imaging techniques, it was possible to observe photo-bleaching of individual fluorophores and to count the steps of bleaching as a measure of the stoichiometry of each CRAC channel complex. We conclude that the subunit stoichiometry in an active CRAC channel is four Orai1 molecules and two STIM1 molecules. Fluorescence resonance energy transfer experiments also showed that four Orai1 subunits form the assembled channel. From the fluorescence intensity of single fluorophores, we could estimate that our transfected HEK293 cells had almost 400,000 CRAC channels and that when intracellular Ca2+ stores were depleted, the channels clustered in aggregates containing about 1,300 channels, amplifying the local Ca2+ entry.

1683/B137

Stretch-Induced Up-Regulation of Caveolae Formation and SOC Activities in HUVEC.

Y. Katanosaka, K. Naruse; Cardiovascular Physiology, Okayama Univ, Okayama, Japan

We have studied about molecular identities of stretch-activated (SA) channels and the mechanism of Ca2+ influx evoked by mechanical stretch in human umbilical vein endothelial cells (HUVECs). Previously, we showed that a targeting suppression of transient receptor potential 2 (TRPV2) protein expression in HUVEC using a TRPV2-specific morphorino-oligo completely blocked a transient increase of intracellular Ca2+ in response to stretch through the activation of SA channels. Furthermore, after these morphant HUVECs were subjected to 20% uni-axial cyclic stretch at 1 Hz for 1 h, neither a stretch-enhanced stress fiber formation nor a shift in the cell orientation transverse to the strain direction could not be observed. From these results, we concluded that TRPV2 would be a key component of SA channel complex and stretch-induced reorganization of cytoskeletons in HUVEC. Here, we examined the remodeling of Ca2+ responses evoked by uni-axial cyclic stretch in HUVEC by fura2 fluorescence imaging. Before and after the cyclic stretch for 15 min, a magnitude of single stretch-evoked Ca2+ transient did not change. However, the Ca2+ influx through the store-operated Ca2+ channels (SOCs) was significantly increased after the 15min- cyclic stretch. Recent studies have demonstrated that caveolae are microdomains in the plasma membrane and contain functionally organized signaling molecules, including Ca2+ signaling. Immunohistochemistry revealed accumulation of caveolin-1 and TRPCs, some of which serve as SOCs, in caveolae after the cyclic stretch to HUVEC. Electron microscopy confirmed that the incidence of caveolae in HUVEC was increased after the stretch. On the other hands, TRPV2-knocked down HUVECs suppressed the increased SOC activities and caveolae formation after cyclic stretch. Such the up-regulation of SOC activities through stretch-dependent TRPV2 activation might contribute to sustained intracellular Ca2+ increase, which is thought to be a primary etiology of the vascular remodeling, and a potent risk factor of pressure-dependent hypertrophic diseases.

1684/B138

Constitutively Active Calcium Current in T Lymphocytes.

P. Thakur, S. Dadsetan, A. Fomina; Physiology and Membrane Biology, University of California, Davis, Davis, CA

Sustained increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is essential for activation and maintaining of T cell functions such as proliferation and cytokine production. Deregulation of Ca^{2+} signaling leads to a number of human disorders including autoimmune and immunodeficiency diseases. Therefore, elucidation of mechanisms regulating intracellular Ca^{2+} dynamics in T cells is important for developing strategies for manipulation of Ca^{2+}-dependent T cell functions and for treating or alleviating human diseases. Here we report that in human T cells we found a previously uncharacterized Ca^{2+} current that operates at unstimulated conditions and displays properties different than those of store-operated Ca^{2+} release activated Ca^{2+} (CRAC) current. Whole-cell and perforated-patch experiments revealed that both constitutively active and CRAC currents displayed inwardly rectifying current-voltage relationship, positive (> 50 mV) reversal potential, and were dependent on the presence of Ca^{2+} in the extracellular solution. However, when divalent cations were removed from the extracellular solution, the monovalent constitutively active current exhibited time-dependent activation and lack of inactivation, whereas monovalent CRAC current displayed fast time-dependent inactivation. These data indicate that constitutively active and CRAC currents are carried via different channels. Measuring store refilling using fura 2 Ca^{2+} indicator and sequential application of cyclopiazonic acid, a reversible blocker of sarco-endoplasmic reticulum Ca^{2+}-ATPase, revealed that store was partially refilled in the absence of the CRAC channel activity. We speculate that in T lymphocytes the constitutively active Ca^{2+} entry channels may supply Ca^{2+} for maintaining resting cytosolic Ca^{2+} levels and/or for store refilling at unstimulated conditions. Supported by AHA Grant-in-Aid 0755086Y to A.F.F.

1685/B139

Analysis of TRPC1-Interacting Proteins by Tandem Mass Spectroscopy.

T. Lockwich[1], A. Makusky[2], J. Kowalak[2], S. P. Markey[2], I. S. Ambudkar[1]; [1]Molecular Physiology and Therapeutics Branch, NIDCR/NIH, Bethesda, MD, [2]Laboratory of Neurotoxicology, NIMH/NIH, Bethesda, MD

Despite extensive studies, the mechanism(s) involved in the regulation of store-depletion activated channels involving TRPC1 remains unknown. Since interacting proteins could provide insights to this mechanism, this study is directed towards identifying the proteins associated with native TRPC1 using a shotgun proteomic approach. A native anti-TRPC1 antibody was used to immunoprecipitate TRPC1 from solubilized rat brain crude membranes under conditions that have been shown to allow retention of TRPC (TRPC3) function. Proteins in the TRPC1 and control (using rabbit IgG) immunoprecipitates were separated by SDS-PAGE and subsequent gel slices trypsinized. After the extraction of the peptides, the peptide fragments were separated and sequenced using HPLC and MS/MS techniques respectively. Analysis of the sequence data reveals the presence of 75 specific TRPC1-associated proteins in the immunoprecipitated TRPC1 fraction. Functional analysis of the TRPC1-associated proteins reveal interactions with several previously known functional roles of TRPC1 (e.g. Ca2+ signaling) as well as some novel pathways (e.g. vesicle fusion). Western blot analysis reveals that TRPC3 co-immunoprecipitates with TRPC1 from the solubilizate of rat brain crude membrane. Not surprisingly, we found extensive overlap between the two proteomes (based on a comparative survey of these results with our previously published TRPC3 proteome). However, both the TRPC1 and TRPC3 immunocomplexes also had unique protein interactions independent of the other. From these data we conclude that TRPC1 and TRPC3 have unique, independent

interactions in the rat brain as well as a shared proteome which could reflect a heteromeric TRPC1/TRPC3 complex in certain regions of the rat brain.

1686/B140

Regulation of Mouse Transient Receptor Potential Type 6 (TRPV6) Channel by Extracellular pH.

S. Ahn[1], J. Sohn[2], B. Yeh[2]; [1]Department of Pharmacology, Wonju College of Medicine, Yonsei University, Wonju, South Korea, [2]Department of Biochemistry and Institute of Basic Medical Science, Wonju College of Medicine, Yonsei University, Wonju, South Korea

The transient receptor potential type 6(TRPV6) channel is a kind of TRP channels which is present in intestine, skin, kidney, prostate, placenta, pancreas, etc. It is well known to play a key role for transepithelial absorption in intestinal tissues. TRPV6 is highly homologous with TRPV5 and forms heteromultimer with it in the distal collecting duct in kidney. The extracellular pH sensitivity of TRPV5 related with hypercalciuria leading to urinary stone formation has reported, but that of TRPV6 is not known. This is thought to be due to the emphasis of the function of TRPV6 in intestinal tissues because physiologically intestinal pH change is very small. However, the extracellular pH sensitivity of TRPV6 may be important as it is expressed well in skin epithelium and calcium plays a critical role for wound healing and aging in skin the pH of which is variable. We report that in whole-cell patch clamp recording extracellular acidification inhibited mouse TRPV6. We found that mutation of aspartate (D517N, D541N) and tyrosine (Y525F) which are located in two extracellular loops between the fifth and sixth transmembrane segments of TRPV6 decreased the sensitivity of the channel to extracellular acidification. Each of the mutant showed slight changes of the function of TRPV6 by acidification, so we checked with double and triple mutant of these three amino acids. These mutants were more sensitive to extracellular pH. As glutamate 522 of the rabbit TRPV5 is known to be a extracellular pH sensor these data suggest that the mechanism of extracellular pH sensitivity of TRPV6 is different from that of TRPV5.

1687/B141

TRPM7 Stimulates M-Calpain by ROS-Dependent Activation of the Stress Activated Protein Kinases P38 and JNK.

L. Su[1,2], H. Chen[1,2], O. González-Pagán[1,2], J. Xie[3], L. Yue[3], L. Runnels[1]; [1]Pharmacology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ, [2]Graduate School of Biomedical Sciences, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ, [3]Cell Biology, University of Connecticut Health Center, Farmington, CT

TRPM7, a member of the transient receptor potential (TRP) channel family, is a unique calcium and magnesium permeant ion channel with its own kinase domain. We previously showed that overexpression of TRPM7 in human kidney cells (HEK-293) produced cell rounding and a loss of cell adhesion that was dependent upon the calcium-dependent protease m-calpain. The TRPM7 elicited change in morphology was channel-dependent, but occurred without any significant increase in cytosolic free calcium. The objective of our study was to determine the mechanism by which TRPM7 activates m-calpain. Here we show that the TRPM7-dependent loss of adhesion can be blocked by inhibitors of the Stress Activated Protein Kinases (SAPKs) p38 and c-Jun N-terminal kinase (JNK). Consistent with this finding, overexpression of TRPM7 or the kinase-inactive form of the channel-kinase increased levels of activated p38 and JNK. p38 and JNK become activated when cells experience stress and is caused by many different factors, including reactive oxygen species (ROS). Application of inhibitors of mitochondrial-dependent ROS production blocked cell rounding, indicating that TRPM7 overexpression produced cell rounding by stimulating ROS-dependent activation of SAPKs. We conclude that TRPM7-overexpression produced cell rounding through ROS-dependent activation of m-calpain via p38 and JNK.

1688/B142

The C-Terminal Hydrophobic Patch of CFTR Interacts with Cytoskeletal Keratin 18 for Stable Surface Expression.

Y. Duan[1], F. Zhang[1], D. Wang[1], W. Zhang[1], Y. Sun[1], P. Yang[1], J. Cuppoletti[3], H. Wang[2], Z. Wu[2], T. M. Magin[4], P. Huang[1]; [1]Biology, Hkust, Hong Kong, China, [2]Biochemistry, Hkust, Hong Kong, China, [3]Molecular and Cellular Physiology, University of Cincinnati, Cincinnati, OH, [4]Institut für Physiologische Chemie, Bonn, Germany

Recent studies have shown that several proteins bind to the C-terminus of CFTR and affect its cell surface expression and channel gating, but interactions found to date do not account for all the known functions of the CFTR C-terminus, suggesting that as yet unidentified proteins that bind to this region of CFTR modulate the function of the channel. To search for such proteins, we performed yeast two-hybrid assays and identified as a CFTR C-terminus binding protein keratin 18 (K18), an intermediate filament proteins expressed in simple epithelial cells, much like CFTR. The interaction of K18 and CFTR was further confirmed by various approaches in mammalian cells. Significantly, our studies on heterologously expressed and endogenous proteins suggested that K18 increased the surface expression of wild-type CFTR. In addition, we found that K18 binding site in CFTR is the highly conserved "hydrophobic patch" (F1413LVI), which has been previously reported to be crucial to CFTR stability in plasma membrane with unknown mechanism. Furthermore, in in vivo study, the reduction of CFTR expression and CFTR activity were observed in the apical domain of villi of duodenum in K18 knockout mice. These in vitro and in vivo studies together suggest a novel regulatory mechanism for CFTR channel function. Supported by RGC grant HKUST6468/05M and 661008.

1689/B143

Species-Specific Differences in Processing of CFTR-ΔF508.

L. Ostedgaard[1], Q. Dong[1], C. Rogers[1], D. Vermeer[1], Y. Zhang[1], T. Rokhlina[1], A. Kabel[1], M. J. Welsh[1,2,3]; [1]Internal Medicine, College of Medicine, University of Iowa, Iowa City, IA, [2]Molecular Physiology and Biophysics, University of Iowa College of Medicine, Iowa City, IA, [3]Howard Hughes Medical Institute, University of Iowa College of Medicine, Iowa City, IA

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel expressed in epithelia, cause cystic fibrosis (CF). The most common CF-causing mutation deletes phenylalanine at position 508 (ΔF508) in the first conserved nucleotide-binding domain (NBD1). This mutation disrupts CFTR processing so that the mutant protein is retained in the endoplasmic reticulum, preventing maturation and traffficking to the plasma membrane. Because mouse models of CF do not replicate the human disease and pig models are being developed, we asked if mouse and pig CFTR-ΔF508 behave like human CFTR-ΔF508. In contrast to human CFTR-ΔF508, some pig

and mouse CFTR-ΔF508 escaped the ER, matured, and retained partial function when expressed in CF airway epithelia. To determine the mechanism for these species differences, we assessed CFTR maturation by pulse-chase. We found that wild-type CFTR matured similarly in all three species. Maturation of human CFTR-ΔF508 was 1-2%, whereas pig CFTR-ΔF508 maturation was 10 to 15%, and mouse was nearly equivalent to its wild-type counterpart. Thus, the severity of the F508 deletion on maturation was human > pig > mouse. Reduced temperature partially rescues human CFTR-ΔF508 processing. However, pig CFTR-ΔF508 was not temperature-sensitive. To identify the regions in CFTR that contribute to processing, we made chimeras of human and mouse CFTR. We chose mouse CFTR because its sequence is only 78% like that of human, it has a less severe processing defect, and the crystal structures of human and mouse NBD1 are known. When we substituted mouse NBD1-ΔF508 into the human CFTR backbone, processing was equivalent to mouse CFTR-ΔF508. By replacing smaller portions of human NBD1 with mouse sequence, we found that the C-terminus of NBD1 was primarily responsible for the rescue. The crystal structures of human and mouse NBD1 suggest that this C-terminal region lies on the surface of NBD where it is accessible for interaction with other domains in CFTR or with other proteins. Our studies point to specific regions within CFTR that alter the effects of the F508 deletion and may guide strategies for correcting the defect caused by this mutation.

1690/B144

Mapping Oculodentodigital dysplasia-linked genetic mutations in Cx43 to an epidermal phenotype using cell, mouse, and human reference models.

J. Churko[1], Q. C. Shao[1], J. Sampson[2], K. Flanigan[2], K. Swoboda[2], D. W. Laird[1]; [1]Anatomy and Cell Biology, University of Western Ontario, London, ON, Canada, [2]Department of Neurology, University of Utah School of Medicine, Salt Lake City, UT

Oculodentodigital Dysplasia (ODDD) is a pleiotropic disorder caused by mutations in the GJA1 gene encoding connexin43 (Cx43). So far, forty-one Cx43 mutations have been found to cause the classical symptoms of ODDD that include soft tissue fusion of the digits, dental malformations, and craniofacial abnormalities, while two mutations (fs230 and fs260) have been reported to cause the additional morbidity of skin disease. Interestingly, the vast majority of characterized mutants, including the fs260 mutant, exhibit dominant effects on co-expressed Cx43 that translates into a loss of gap junctional intercellular communication. Thus, we hypothesize that the mechanism of action for frame-shift mutants causing an increase in disease load may be distinct from the mutations that result in signal amino acid substitutions. To determine if the fs230 mutant exhibits properties similar to the fs260 mutant, it was engineered and expressed in rat epidermal keratinocytes (REKs). The fs230-GFP mutant was found to dominantly inhibit the passage of Lucifer yellow by 66% and reduce the number of gap junction plaques by 34% when expressed in Cx43-positive REKs. However, the skin phenotype observed in ODDD patients harboring frame-shift mutations cannot be explained solely by the reduction in Cx43 gap junctions as the epidermis from a mutant mouse harboring the G60S mutation and from an ODDD patient skin biopsy, revealed a similar reduction in Cx43 gap junctions but a skin phenotype was not evident. In ongoing experiments designed to further assess the unique properties of frame-shift mutants in skin disease, both frame-shift and selected missense mutants were expressed in differentiation-competent REKs to assess their impact on keratinocyte proliferation and differentiation. Our results to date would suggest that the added burden of skin disease in ODDD patients may be linked to mechanisms associated with the sequestering of Cx43 within intracellular compartments. This work was funded by the CIHR.

1691/B145

The Role of PKC-Γ in Regulating Lens Gap Junction Coupling.

S. Das[2], F. Martinez-Wittinghan[1], H. Wang[1], D. J. Takemoto[2], G. Zampighi[3], R. T. Mathias[1]; [1]Physiology & Biophysics, SUNY at Stony Brook, Stony Brook, NY, [2]Biochemistry, Kansas State University, Manhattan, KS, [3]Neurobiology, UCLA, Los Angeles, CA

In the lens, activation of PKC-γ with phorbol esters has been reported to phosphorylate connexins and down regulate gap junction coupling. To determine whether PKC-γ has a role in regulation of gap junction coupling in the normal lens, we have compared the properties of coupling in lenses from wild type (WT) and PKC-γ knockout (KO) mice. Gap junction protein expression was studied using Western blotting; gap junction structure was observed using thin section and freeze fracture electron microscopy, and conical tomography; gap junction coupling conductance was measured in intact lenses using impedance studies. There were no significant differences in size or clarity or expression of Cx46 or Cx50 in lenses from WT and PKC-γ KO mice. However, in WT lenses, Cx43 is found only in epithelial cells whereas in PKC-γ KO lenses, expression continues into the fiber cells, with the highest levels in the outer differentiating fibers (DF) but some detectable protein in the inner mature fibers (MF). DF and to some extent MF of PKC-γ KO lenses contained vesicular like structures, formed from two membranes connected by a gap junction. These structures could be invaginations of cell to cell junctions that were sectioned and thus appeared vesicular, or internalized junctions on their way to degradation. In either case they were never seen in WT lenses and represent a large increase in the area of gap junctions over that seen in WT lenses. Gap junction coupling conductance in the DF of PKC-γ KO lenses was 19% larger than that of WT. In the MF, the effect was much larger with the KO lenses having a 253% increase in coupling over WT. In summary, PKC-γ has a major role in the regulation of gap junction expression and coupling in the normal lens. The largest effects of PKC-γ KO on Cx43 expression and cellular structure occurred in the DF whereas the largest increase in coupling conductance occurred in the MF, where Cx46 is thought to solely mediate coupling. Perhaps Cx46 is normally phosphorylated by PKC-γ, which inhibits the number of open channels. (Ey06391; EY13421; EY04410; Kansas City Area Life Sciences Institute.)

1692/B146

Immunolocalization of Mitochondrial ATP-Dependent K+ Channel Subunits in Human Osteoblast-Like Cells.

J. Butler[1], A. Warley[2], K. Brady[2], F. McDonald[1]; [1]Department of Orthodontics, King's College London, London, United Kingdom, [2]Centre for Ultrastructural Imaging, King's College London, London, United Kingdom

The mitochondrial ATP-dependent K+ channel (mitoKATP) has been shown to play a role in cellular protection against apoptosis, or programmed cell death. This channel has been identified and characterized in a number of cell and tissue types but to date the possible existence of mitoKATP in osteoblastic cells has not been investigated. Osteoblast apoptosis is integral to the control of bone turnover, and the recently described effects of nitric oxide (NO) and Akt (protein kinase B) on osteoblast cell survival suggest a possible role for mitoKATP as a regulatory mechanism for apoptosis in osteoblasts. The aim of this investigation was to establish whether the mitochondria of human

osteosarcoma-derived osteoblasts (SaOS-2 cells) contain the putative mitoKATP subunits Kir6.1 and Kir6.2. Ultrathin thawed cryosections of SaOS-2 cells were prepared for transmission electron microscopy using an adaptation of the method described by Tokuyasu, and immunolabelled using goat anti-Kir6.1 or anti-Kir6.2 antisera as the primary label, and a 10 nm colloidal gold-conjugated donkey anti-goat secondary antibody. The effectiveness of the antisera and of the immunolabelling protocol were confirmed by using a sample of rat cardiac muscle as a positive control. Ultrastructural examination by transmission electron microscopy revealed that SaOS-2 cells contain Kir6.2 but not Kir6.1, and that Kir6.2 is present in the mitochondrial inner membranes under experimental conditions, but at extremely low density. These findings suggest that human osteoblast-like cells might contain mitoKATP channels in which Kir6.2 is the pore-forming subunit, although it appears that these channels are likely to be present in extremely low abundance under basal conditions.

1693/B147

The Localization and the Trafficking of Cardiac Potassium Channel SK2 and L-Type Calcium Channel Cav1.3 Complex Affected by Its Interacting Partners.

L. Lu, V. Timofeyev, A. Singapuri, N. Chiamvimonvat; Department of Internal Medicine, University of California, Davis, CA

The specific sub-cellular localization and protein-protein interactions are critical in the proper function of cardiac ion channels. Abnormalities in the functions of ion channel proteins can result in the alteration of cardiac refractory period and increased occurrences of arrhythmias. The trafficking of ion channels is only beginning to be elucidated, and the entire trafficking pathway is not known for any single channel. Our laboratory has previously documented the important functional roles of a small conductance Ca2+-activated K+ channel (SK2 channel) in human and mouse atrial myocytes. Moreover, we have shown that the interaction of SK2 channel with alpha-actinin2 localizes the channel to L-type Ca2+ channel (Cav1.3) which mediates the entry of external Ca2+ source to regulate the channel function. Here, we directly demonstrate that trafficking of cardiac SK2 channel is dependent on its interaction protein, alpha-actinin2. Expression of cardiac SK2 channel alone in HEK293 cells results in the localization of SK2 channel mostly in the cytosol of the cells. However, co-expression of cardiac SK2 channel with alpha-actinin2 aids in the proper trafficking of the channel to the plasma membrane. The binding sites on SK2 channel with its interacting partners, alpha-actinin2 was elucidated using site-directed mutagenesis and yeast-two-hybrid assays. Mutations of T431 and W432 residues on the C-terminus of SK2 channel to alanine completely suppress the interaction between SK2 channel and alpha-actinin2 protein. Immunofluorescence imaging data further suggest that proper SK2 channel membrane localization may require cytoskeleton anchoring by alpha-actinin2 as well as endocytic recycling via early endosome (EEA) back to the cell membrane. Finally, we further demonstrate that the localization pattern of SK2 channels in atrial myocytes is critically dependent on the normal expression of Cav1.3 channels. Genetic ablation of Cav1.3 channel in a mouse model results in abnormal distribution of SK2 channel and prolongation of the atrial repolarization and atrial arrhythmias.

1694/B148

Charge Transfer of Cav2.2 Channels during G-Protein Modulation in Rat Sympathetic Neurons.

S. Rebolledo-Antunez, J. M. Farías, I. Arenas, D. E. Garcia; Facultad de Medicina, Universidad Nacional Autonoma de Mexico, Mexico City, Mexico

The N-type ($Ca_V2.2$) calcium channels are modulated by a wide range of neurotransmitters through G-protein coupled receptors. It has been suggested that G-protein voltage-dependent modulation is carried out at the closed states transiting the channel toward its opening. The transition between these states requires charge transfer of the voltage sensor. In a previous work we demonstrated that G-protein activation decreases intramembrane charge movement in rat sympathetic neurons (Am J Physiol Cell Physiol 292: C2226-C2238, 2007). The purpose of this study was to estimate the number of gating charges of $Ca_V2.2$ channel in control and G-protein modulated conditions. In this work, to demonstrate that $Ca_V2.2$ channels visit several closed states before opening, we applied the Cole-Moore protocol. The observed shift indicates that $Ca_V2.2$ goes through a series of closed states before channel opens. If G-protein voltage-sensitive modulation is carried out at these closed states, we expect that there would be a greater Cole-Moore lag in the presence of neurotransmitter. This prediction was confirmed for norepinephrine. By contrast, in the presence of angiotensin II there was not a significant change on the delay of $Ca_V2.2$ channel activation. We used the limiting slope method for calculation of the gating charge per channel. The evaluated effective charge z was 3.78 ± 0.24 e_0 for $Ca_V2.2$ channels. To determine whether G-protein inhibition alters charge transfer, we calculated gating charge per channel using GTPγS. This procedure reduces elementary charge by ~2 e_0. To discriminate between voltage-sensitive and voltage-insensitive inhibition we used specific neurotransmitters. In the presence of angiotensin II, gating charge per channel remains unchanged with respect to GDPβS and control, while with norepinephrine there is a gradual decrease depending on its concentration to reach the value of ~2 e_0, suggesting that the reduction is due exclusively to the G-protein voltage-sensitive modulation. Therefore, we conclude that there is a significant charge transfer decrease of $Ca_V2.2$ channels, which depends on the acting G-protein inhibition pathway. Supported by UNAM-DGAPA-PAPIIT IN200407

1695/B149

Characterization of a Novel Macrophage Endosomal Splice Variant of the Human SCN5A Sodium Channel.

E. A. Jonas[2], L. M. Carrithers[1], G. Chatterjee[3], M. D. Carrithers[1]; [1]Neurology, University of Wisconsin School of Medicine, Madison, WI, [2]Internal Medicine, Yale University School of Medicine, New Haven, CT, [3]Neurology, Yale University School of Medicine, New Haven, CT

Recent work from our laboratory demonstrated expression of voltage-gated sodium channel variants (SCN5A and SCN8A) in monocyte-derived macrophages and the human monocytic cell line, THP-1. One of these channels, NaV1.5, which is encoded by SCN5A, is expressed on the macrophage late endosome to regulate endosomal acidification. Here we further characterized the SCN5A macrophage variant by cDNA cloning and patch clamp analysis. Sequence analysis revealed expression of a full length transcript variant of human SCN5A that has a deletion in exon 25, which encodes a portion of the selectivity filter. Patch clamp recordings of endosomes isolated from differentiated and immune activated THP-1 cells revealed single channel activity of variable conductance that lacked clear voltage dependence or selectivity. Channel activity was increased in frequency by the specific sodium channel agonist veratridine, and patch activity was inhibited by amiloride. Isolated endosomes from cells in which NaV1.5 had been knocked down or endosomes that contained TTX were deficient in veratridine-sensitive activity. The macrophage splice variant of SCN5A also was analyzed following over-expression in HEK-293 cells.

Preliminary data suggest that these transfected 293 cells contain channel activity in the plasma membrane similar to that recorded in isolated endosomes. These results suggest that human macrophage SCN5A is a novel spice variant that encodes a variant of the NaV1.5 sodium channel with unique electrophysiological properties and subcellular function. This work is supported by awards from the National Multiple Sclerosis Society, RG-3888, and the NIH, NINDS K02 NS047457-01, to M.D.C.

1696/B150

Sodium Channel Subtypes Switch Subcellular Localization between Nucleus and Cytoplasm in Hippocampal Neurons and PC12 Cells.

M. Longart, E. Gomez, L. Garcia, O. Felizzola, C. Malave; Unidad de Neurobiologia. Centro de Biociencias y Medicina Molecular, Instituto de Estudios Avanzados IDEA, Caracas, Venezuela

Na channel are implicated in the generation and transmission of electrical signals in the nervous system. They regulate the establishment of neuronal networks during development and are involved in the regulation of neuronal polarity. The subcellular localization of Na channels from their synthesis to their final destination in the cell is largely unknown. Since the location of the channels is very important to determine their particular functions we studied their distribution in PC12 cells and hippocampal neurons, by immunofluorescence and cellular fractionation. Initial experiments using conventional wide field fluorescence microscopy and a generic sodium channel antibody (anti Pan-Nav) in PC12 cells showed an unexpected nuclear localization. To confirm this result, we prepared nuclear and cytoplasm fractions from these cells and performed Western blot analysis. We observed a different distribution of several Na channels type between nucleus and cytoplasm in PC12 cells. Additionally, immunofluorescence of hippocampal neurons with antibodies against different sodium channel types also showed a different subcellular location. Confocal analyses of hippocampal neurons cultures, double labeled with DAPI and anti Pan-Nav, showed that sodium channels are surrounding the nucleus but not inside it. Experiments to determine the specific subcellular compartment (nuclear membrane, endoplasmic reticulum, Golgi, etc.) where the channels localize are presently in course. These results show a new location for sodium channels. The determination of the function of the nuclear location of these proteins is key to understand important aspects of neuronal biology as neuronal differentiation, polarization and, excitability.

1697/B151

Functional Characterization of a Vesicular Monoamine Transporter (VMAT2) Expressed in Sf9 Cells.

Y. Elbaz, T. Danieli, S. Schuldiner; Biological Chemistry, The Hebrew University of Jerusalem, Jerusalem, Israel

The vesicular monoamine transporter (VMAT2/SLC18A2) catalyzes storage of monoamines—serotonin, dopamine, norepinephrine, epinephrine, and histamine in synaptic vesicles and the storage granules of secretory cells. The transport of monoamines by VMAT2 involves exchange of 2H+ with one substrate molecule at the expense of the proton gradient formed by the V-type ATPase . VMAT2 is an essential protein mainly expressed in the central nervous system. In addition to the native substrates, VMAT2 interacts with many clinically relevant drugs, including the psychostimulants 3,4-methylenedioxymethamphetamine (MDMA) and amphetamines and the parkinsonian toxin 1-methyl-4-phenylpyridinium (MPP+). Moreover, recent publications indicate VMAT2 as a newly emerging target for both diagnostic and therapeutic applications in diabetes mellitus. There is lack of biochemical and structural data regarding vesicular transporters due to the difficulties in their expression and analysis of their function. Heterologous over-expression of mammalian membrane proteins in general is still a major bottleneck on the way to decipher their mechanism. We present the optimized over-expression of VMAT2 using baculovirus expression system to amounts large enough for biochemical and structural studies (1-2mg/liter). We have functionally characterized the protein and found its pharmacological profile to be similar to that of the native protein. The expressed protein binds the clinically used drug tetrabenazine which is a known noncompetitive inhibitor. Upon reconstitution to proteoliposomes, the protein catalyzes [3H]-5HT uptake in a pH dependent manner, displays wild type affinity for serotonin, and its turnover rate is similar to that shown in native systems (~0.4 molecule/sec). The protein was further purified and characterized using spectroscopic tools.

Protein Folding and Assembly (1698 – 1711)

1698/B159

Localization Dependent Degradtion of Misfolded Proteins.

R. Prasad, D. Ng; A/Prof Davis Ng's Group, TLL,NUS, Singapore.

The endoplasmic reticulum (ER) is a major site of protein synthesis in eukaryotic cells. The ER contains a system called ER quality control (ERQC). ERQC function to retain terminally misfolded proteins, which are otherwise harmful to the cell. Those misfolded proteins are then targeted to the ER-associated protein degradation (ERAD) machinery. There are two sequential checkpoints present in ER that recognize topologically distinct domains of polypeptides. The first checkpoint monitors the cytosolic domains of membrane proteins and the second checkpoint monitors lumenal domains and soluble proteins. Proteins detected by either checkpoint are degraded by ERAD. We have previously identified that, at the ER, ERAD-C pathway monitors the folding state of cytosolic domains of membrane proteins and rapidly clears detected proteins from the ER. We have demonstrated that when the misfolded cytosolic domains of membrane proteins were untethered from the ER-membrane, they are now recognized by completely different quality control mechanism. Here we have identified that the folding states of proteins in or facing the cytosol are monitored by different quality control system. Though location of lesion present in a misfolded protein is important as a determinant for the pathway by which it degrades, its spatial localization is also very important. Key Words ERQC, ERAD. Reference Vashist, S., and Ng, D. T.(2004). Misfolded proteins are sorted by a sequential checkpoint mechanism of ER quality control. J Cell Biol 165, 41-52.

1699/B160

Loss of Specific Chaperones Involved in Membrane Glycoprotein Biosynthesis during the Maturation of Human Erythroid Progenitor Cells.

S. Patterson[1], J. Li[1], J. Kang[2], A. Wickrema[2], D. Williams[1], R. Reithmeier[1]; [1]Biochemistry, University of Toronto, Toronto, ON, Canada, [2]Medicine, Section of Hematology/Oncology, University of Chicago, Chicago, IL

The production of erythrocytes requires the biosynthesis of major red cell membrane glycoproteins, Anion Exchanger 1 (AE1) and Glycophorin A (GPA) that involves the endoplasmic reticulum (ER), glycoprotein translocation, N-glycosylation, and quality control machinery. We found that there was a complete loss of calnexin, an ER glycoprotein chaperone, and ERp57, a protein disulfide isomerase, as well as a major decrease of the cytosolic chaperones, Hsc70 and Hsp70, as AE1 and GPA are produced, late in the differentiation of human CD34+ erythroid progenitor cells in culture. In contrast, the levels of other chaperones (BiP, calreticulin and Hsp90), components of the protein translocation (sec61α) and glycosylation machinery (OST48), and cytosolic proteins (actin and GAPDH), were maintained. Calnexin did not exhibit a prolonged interaction with misfolded AE1 mutants expressed in HEK cells and disruption of calnexin-glycoprotein interactions in K562 cells using castanospermine did not affect the cell surface levels of endogenous GPA or expressed AE1. These results demonstrate that calnexin and ERp57 are not required for the synthesis of major membrane glycoproteins (AE1 and GPA) during the terminal differentiation of human erythroid precursors.

1700/B161

Blocking Substrate Ubiquitination Rescues the Degradation of ERAD Substrates and Results in Trafficking from the ER.

K. J. Hill[1], D. Stringer[2], R. Piper[2], A. Cooper[1]; [1]Diabetes Program, Garvan Institute of Medical Research, Sydney, NSW, Australia, [2]Department of Physiology, University of Iowa, Iowa City, IA

In the ER of wild-type cells, the membrane protein Vph1 is stably assembled with other integral membrane subunits into the Vo sector of the H+-ATPase complex that functions to acidify the lysosome-like vacuole. Cells lacking the ER-localized V-ATPase assembly complex, Vma12/21/22, fail to assemble the Vph1-containing Vo sector and Vph1 instead remains in the ER and is degraded. Surprisingly the degradation of unassembled Vph1p is independent of the ubiquitin ligases HRD1 and DOA10 typically associated with ERAD. Screening of all potential ubiquitin ligases found that Rsp5p (a HECT-type E3 ligase and Nedd4-family member) is responsible for ubiquitination of unassembled Vph1. To examine the role of Vph1 ubiquitination in its turnover and trafficking, we studied a fusion protein of Vph1 and the catalytic domain of a deubiquitinating enzyme. The resulting Vph1, which is resistant to ubiquitination, was resistant to the degradative and trafficking defects associated with unassembled Vph1. Vph1-DUb assembled correctly into the V-ATPae complex in wild-type cells but failed to be degraded when unassembled in vma22 cells. The stabilised unassembled Vph1-Dub did not remain in the ER and instead was surprisingly found on the plasma membrane, while normally assembled Vph1-Dub correctly localized to the vacuolar membrane. These and other studies indicate that ubiquitin may act bifunctionally not only as a signal for eventual proteasomal degradation but also as a means of retaining misfolded membrane proteins at the ER to promote their entry into an ERAD pathway.

1701/B162

Quality Control of Non-Glycosylated Proteins.

S. Wang, D. Ng; Temasek LifeSci Laboratory, National Uni of Singapore, Singapore, Singapore

The term quality control describes mechanisms that ensure only properly folded and assembled proteins are produced. ER-associated degradation (ERAD) targets irreversibly misfolded and unassembled proteins for destruction and is classified into ERAD-L (luminal) and ERAD-C (cytosolic). The term quality control describes mechanisms that ensure only properly folded and assembled proteins are produced. ER-associated degradation (ERAD) targets irreversibly misfolded and unassembled proteins for destruction and is classified into ERAD-L (luminal) and ERAD-C (cytosolic). Proteins with a misfolded luminal domain are targeted to the ERAD-L pathway whereas proteins harboring lesions in the cytosolic domain are degraded via the ERAD-C pathway. ERAD-L relies on the placement of specific N-linked glycans as part of the signal for degradation. This raised the interesting question of how secretory pathway proteins that lack N-linked glycans are monitored for folding. In this study, we focused on the quality control mechanisms of a non-glycosylated yeast transmembrane protein. Various mutations were introduced into its luminal domain and the resulting mutant proteins behaved similarly. They were all misfolded and rapidly degraded in the wild-type cells. Currently, we are testing whether the degradation relies on some known ERAD components. So far, the results indicate the proteins were degraded independently of Yos9p and Htm1p, two factors involved in the quality control of glycosylated ERAD substrates. We propose that the cells employ a specific degradation pathway for non-glycosylated proteins and such a mechanism is probably conserved from the yeast to the mammalian cells.

1702/B163

The Derlin-1/Hrd1 Complex Facilitates Cholera Toxin Retro-Translocation.

K. B. Dezsi, M. Forster, B. Tsai; Cell and Developmental Biology, University of Michigan, Ann Arbor, MI

Cholera toxin (CT) intoxicates cells with its receptor binding B subunit (CTB), allowing for cell entry and trafficking to the endoplasmic reticulum (ER), where the catalytic A1 subunit (CTA1) is recognized as a substrate of ER-associated degradation (ERAD). CTA1 is consequently retro-translocated into the cytosol to allow for a signaling cascade leading to diarrhea. How CT is targeted to the site of retro-translocation in the ER and the identity of ERAD membrane components involved this process are unknown. Using a semi-permeabilized, cell-based retro-translocation assay, we demonstrate that expression of the dominant negative Derlin-1-YFP fusion protein and catalytically inactive Hrd1-C291A mutant each inhibit the retro-translocation of CTA1. Co-immunoprecipitation studies revealed an interaction between CTB with both Derlin-1 and Hrd1. An in vitro membrane-binding assay demonstrated that CTB stimulates the binding of CTA1 to the ER membrane. Finally, intoxicating cells with CTB stabilized a Derlin-1-dependant substrate, supporting the idea that CT utilizes the Derlin-1 pathway. These findings demonstrate a role for Derlin-1 and Hrd1 in the transport of CTA1 from the ER to the cytosol, and suggest a role for CTB in targeting the holotoxin to the ER membrane retro-translocation components.

1703/B164

Erasin Recruits P97/VCP and Ubiquilin to the ER during ER-associated Degradation.

R. Danner, J. Liang, H. Doong, C. Harman, H. Wang, S. Fang, M. J. Monteiro; MBC, University of Maryland Biotechnology Institute, Baltimore, MD

The endoplasmic reticulum (ER) is the organelle where over one-third of all proteins in cells are synthesized and where quality control systems operate to ensure that only correctly folded proteins and assembled complexes are allowed to exit the site for delivery to their sites of function. A build up of misfolded proteins in the ER leads to ER stress, which has been linked to many human diseases. Terminally misfolded proteins are cleared from the ER by a process called ER-associated degradation (ERAD), a highly regulated process by which misfolded proteins in the ER are retranslocated from the ER to the cytosol or nucleoplasm for degradation by the proteasomes. The composition of the ERAD complexes in mammalian cells is still poorly understood. Here, we describe three proteins that form a complex that we propose functions in ERAD in mammalian cells. The complex is composed of erasin, a ubiquitin-regulatory-X domain-containing protein that promotes ERAD, the molecular chaperone, p97/VCP, and ubiquilin, a protein that is thought to act as a shuttle factor that delivers misfolded proteins to the proteasomes for degradation. We demonstrate that purified erasin, p97/VCP, and ubiquilin proteins bind together to form a trimeric complex, with erasin serving as a platform for p97/VCP and ubiquilin binding. We confirmed the presence of the complex by coimmunoprecipitation of each of the proteins with one another. Functionally, we demonstrate that reduction of erasin or ubiquilin expression results in reduced degradation of the ERAD substrates, CD3delta and alpha-1-antitrypsin. These results suggest that erasin, ubiquilin and p97/VCP form a novel complex involved in ERAD.

1704/B165

Active Interleukin-31 Is Secreted as Disulfide-Bonded Covalent Aggregates: Role of Conserved Cysteine Residues in Aggregate Formation, Intracellular Trafficking, and Secretion.

S. Byrd, N. Patel, M. Shen, K. H. Edelmann, S. Brouns, C. Mehlin, D. P. Meininger, H. Hasegawa; Protein Science, Amgen Inc., Seattle, WA

The quality control mechanism of the ER has been well established in a variety of cellular models. In our larger effort to characterize protein biosynthesis of therapeutically important secretory cargoes, however, we occasionally encounter secretory proteins that are at odds with this paradigm. One such example is a potent inflammatory cytokine, interleukin-31 (IL-31). Recombinant IL-31 is secreted as intermolecularly disulfide-bonded aggregates. Despite the extent of covalent aggregation, both secreted and intracellular pools of IL-31 remained soluble. The secreted aggregate not only binds to its cognate receptor, OSMR/IL-31R heterodimer, but it activates the receptor in a cell-based assay system. The aggregation of recombinant mu IL-31 was independent of promoter strength. The aggregate formation was not caused by an intrinsic limitation in the folding capacity of the ER because co-expression of XBP1(S) served only to boost protein secretion (~6-fold) without reducing the level of aggregation. At steady state, the intracellular pool of IL-31 localized predominantly to the Golgi compartment, suggesting that the ER quality control system does not actively retain the aggregate in the ER. Appending a KDEL motif to the C-terminus both increased ER retention and reduced the secretion of IL-31. The ER-retained IL-31-KDEL forms the characteristic aggregates, suggesting that they are emerging at a pre-Golgi stage. While the predicted mature N-terminus is Thr-24, the actual N-terminus was Ala-31, leaving 5 cysteine residues in the mature protein, instead of 6. When fused to an exogenous signal peptide, the 6-cysteine version of mu IL-31 was retained in the ER and poorly secreted, whereas the 5-cysteine form was secreted efficiently as aggregates, suggesting that a precise signal cleavage event is coupled to the regulation of protein maturation and secretion. Disulfide mapping studies identified a putative reactive unpaired cysteine. To provide biochemical basis for the aggregate formation and secretion, we performed a systematic cysteine mutagenesis to evaluate their effects on protein maturation. A potential mechanism of aggregate formation and the roles of individual cysteine residue will be discussed.

1705/B166

Identification of Genes with Genetic Interactions with the Unfolded Protein Response Transducer, Ire1p.

N. Ismail, D. Ng; Temasek Life Sciences Laboratory, Singapore, Singapore

The endoplasmic reticulum (ER) is a major site of protein biogenesis in the cell. Perturbations to the cell, such as genetic mutation or environmental changes, may result in the accumulation of misfolded or unfolded proteins within the ER lumen, causing ER stress. This triggers a signaling cascade known as the unfolded protein response (UPR) which serves to restore ER homeostasis. The main UPR transducer in *Saccharomyces cerevisiae* is the ER membrane protein, Ire1p. Ire1p senses the presence of these misfolded/unfolded proteins and activates *HAC1*, which in turn upregulates the expression of about 381 genes to restore ER balance. In order to better understand the physiology of the UPR, we seek to identify genes with genetic interactions with *IRE1*. In this study, a genetic approach exploiting yeast colony colour phenotype was used to identify genes which, when deficient, render the UPR essential for viability. Some of the genes identified in this screen include *SCJ1*, *LHS1*, *PDI1*, *UBC7* and *MGA2*. These factors have roles in protein folding (such as DnaJ protein Scj1p, Hsp70 protein Lhs1p and Pdi1p), ER-associated degradation (Ubc7p) and the regulation of lipid synthesis (Mga2p) - all of which are functions associated with the ER. This genetic strategy provides a powerful tool to discover novel genes involved in various aspects of protein biogenesis in the secretory pathway.

1706/B167

A Receptor Possessing Chaperone Activity: the Sigma-1 Receptor at the Mitochondrion-Associated ER Membrane.

T. Hayashi, T. Su; Cellular Pathobiology Section/CNRB/IRP, National Institute on Drug Abuse/NIH/DHHS, Baltimore, MD

Protein misfoldings under cellular stress yield highly toxic aggregates that are implicated in the pathogeneses of several human diseases including neurodegenerative disorders, cancer, and diabetes mellitus. Molecular chaperones are known to be, in response to stress, rapidly upregulated to correct the protein misfolding. Molecular chaperones can also regulate signal transductions by participating in the coupling of multiple signaling molecules. Thus, molecular chaperones play important roles in cell survival as well as in signal transduction, and may therefore represent potential therapeutic targets for human diseases. However, pharmacological approaches targeting molecular chaperones are difficult and have emerged just very recently. In this study, we discovered that a receptor can possess the chaperone activity, thus enabling the activation/deactivation of chaperones via pharmacological means at will. The sigma-1 receptor (Sig-1R) at the endoplasmic

reticulum (ER) is found by us to be such a receptor. The Sig-1R, possessing two membrane-spanning regions, localizes exclusively at the ER subdomain directly apposing the mitochondrial outer membrane (i.e., the mitochondrion-associated ER membrane). The C-terminus of the Sig-1R at the ER lumen exerts potent activity against protein aggregation. Sig-1Rs form complexes with BiP in the presence of high [Ca2+]ER. The depletion of [Ca2+]ER however dissociates BiP from the Sig-1R which in turn unleashes its chaperone activity on the type-3 IP3 receptors at the ER-mitochondrion contact, thus ensuring proper Ca2+ transmission from the ER to mitochondria. Interestingly, Sig-1R agonists, which promote neuronal survival in the brain, cause the dissociation of the Sig-1Rs from BiP in a Ca2+-independent manner. This action of agonists is blocked by selective Sig-1R antagonists. Thus, the sigma-1 receptor chaperone, whose activity can be activated/inactivated by specific ligands including antidepressants, steroids, and benzomorphans, represents a novel pharmacological target for several human diseases. (Supported by IRP/NIDA/NIH/DHHS)

1707/B168

Dopamine Selectively Activates the ER-Stress Sensor IRE1.

T. Mori, T. Hayashi, T. Su; Cellular Pathobiology Section, NIDA/IRP/NIH/DHHS, Baltimore, MD

Dopamine (DA) is known to induce cytotoxicity which involves oxidative stress. In this regard, the DA-induced cytotoxicity has been implicated in the mitochondrial dysfunction as it relates to the Parkinson's disease. However, the similar action of DA on other cellular organelles such as the endoplasmic reticulum (ER) is less well understood. Oxidative stress is known to induce the unfolded protein response (UPR) at the ER which encompasses the activation of three UPR transducers at the ER, ATF6, IRE1 and PERK that in turn increase the transcription of genes including chaperones. In this study we used CHO cells and examined if DA may activate the UPR transducers and, if any, the resultant increase of chaperones including the sigma-1 receptor (Sig-1R) which was recently shown by us to be a new, novel ER chaperone forming a dynamic complex with another chaperone BiP (Hayashi and Su, Cell, 131:596, 2008). We found here that, as expected, the ER Ca2+ uptake blocker thapsigargin (0.3 μM) activates all UPR transducer proteins. However, DA, at a comparable concentration of 10 μM in the synaptic region, increases only the activation of IRE1 (i.e., increase of phosphoIRE1), but not that of PERK or ATF6. Indeed, we found that 10 μM of DA does not significantly change the levels of downstream signaling molecules purportedly incurred upon the activation of PERK (e.g. ATF4, CHOP). DA (10-1000 μM; 1hr) causes an increase of Sig-1Rs by 200% as well as an increase of BiP. Further, we show that the DA-induced activation of IRE1 and the increases of Sig-1Rs and BiP are all blocked by free radical scavengers. These results indicate that DA causes the ER stress which is specifically sensed by the UPR transducer IRE1 and that Sig-1Rs and BiP represent cellular responsive elements to counteract the deteriorating action caused by DA. (This study is supported by the Intramural Research Program of the NIDA, NIH, DHHS)

1708/B169

Two Conformations of a Chimeric Yeast Prion Exhibit Large Scale Differences.

C. Foo[1], J. Weissman[1,2]; [1]UCSF, San Francisco, CA, [2]HHMI, San Francisco, CA

The yeast prion *[PSI+]* is a useful model for studying aspects of prion behavior such as strains and species barriers. A strong species barrier separates the *[PSI+]* prions from *Saccharomyces cerevisiae* and *Candida albicans*. A chimera of the two prion proteins, however, is able to interact with either species. This ability is due to the chimera's formation of two distinct strains. We hypothesize that these strains must contain large-scale conformational differences since the two strains exist on opposite sides of a species barrier. To study these differences, we use hydrogen-deuterium exchange on prion fibers and assess the exchange by NMR. Preliminary results suggest that the structural cores of the two strains largely do not overlap. In particular, the N-terminal region of the protein, which is known to be highly structured and essential for the prion behavior of *[PSI+]* in *S. cerevisiae*, is less protected in the *C. albicans*-compatible strain. This indicates the wide extent of conformational diversity that exist in prions, and the strength of the conformational barrier to interspecies transmission of prions.

1709/B170

Structural Elucidation of the MBP (Maltose Binding Protein) C-Termini from Fusion Proteins in Pichia Pastoris Expression System.

G. Lin-Cereghino[1], Z. Li[2], W. Leung[1], S. Gomez[1], A. Franz[2], J. Lin-Cereghino[1]; [1]Biological Sciences, University of the Pacific, Stockton, CA, [2]Chemistry, University of the Pacific, Stockton, CA

Pichia pastoris is widely used for heterologous protein expression. Over 700 proteins from bacterial to human sources have been produced in this yeast. However, some foreign proteins, which are engineered to be secreted, are retained inside the cell. Secondly, more fusion peptides are needed to assist in the purification of proteins made in P. pastoris. Our objective is to use E.coli Maltose Binding Protein (MBP) as an "escort" protein to improve secretion and purification of recombinant proteins from P. pastoris. To determine if MBP can be used as a temporary "escort" protein for secretion and purification, a series of fusion proteins were prepared in P. pastoris. MBP was fused at the N-terminus to human FKBP12, a protein that is difficult to produce in sufficient quantity from P. pastoris. However, western analysis indicated that only the MBP portion was secreted efficiently into the culture medium, which suggested that the fusion protein had been proteolyzed between MBP and FKBP12. We hypothesized that an unknown protease inside the cell had cleaved the fusion protein at an unspecific site between the two peptides, perhaps at the Factor Xa sequence (IEGR). We created several mutations in the IEGR region and utilized mass spectrometry to identify the precise amino acids that had been cleaved. Mass spectrometry analysis with LTQ XL indicated that in the original fusion peptide, the MBP was proteolyzed after the R in the Factor Xa sequence. We used site-directed mutagenesis to create three other MBP-FKBP12 fusion proteins with modifications in or around the IEGR sequence. We are in the process of expressing new MBP-FKBP12 mutants and using mass spectrometry to determine the sequence of their C-termini. Our goal is to determine the protease cleavage sites and, in the future, identify the responsible protease within the yeast secretory network.

1710/B171

Expression and Regulation of Na+/H+ Exchanger 3 by B-Pix - Shank2 Protein Complex.

J. Lee, M. Lee; Department of Pharmacology and Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul 120-752, South Korea

Na+/H+ exchanger 3 (NHE3) plays an important role in neutral Na+ transport in the mammalian epithelial cells. Therefore, it is necessary to more accurately define their relative contributions to Na+ transport for an efficient homeostasis. It was found that expression and activity of NHE3 was regulated by PDZ (PSD-95/discs large/ZO-1) domain-based adaptor, shank2. The mechanism for the regulation of NHE3 by Shank2, however, is not known. Since the membrane stability of ion transporter is regulated by Rho small GTPase, we further investigated the regulation of NHE3 activity by Shank2 in relation to β-Pix which is a strong binding partner to shank2 and a guanine nucleotide exchange factor for the small GTPase. When expressed in PS120/NHE3 cells, β-Pix increased the membrane expression and basal activity of NHE3. On the other hand, this phenomenon was abolished by mutations in the PDZ domain of Shank2. Interestingly, the small GTPase inhibitor Toxin B abolished β-Pix-induced increases of NHE3 membrane expression. The knock-down of endogenous β-Pix expression by RNA interference decreased shank2-induced increase of NHE3 membrane expression. In addition to its PDZ domain, shank2 contains a sam domain which is responsible for multimerization of shank2. The dominant negative form of shank2 sam domain attenuated significant for cross-linking multiple sets of protein complexes with the NHE3 and β-Pix. Furthermore, β-Pix was physiologically associated with NHE3-shank2 in rat tissues such as colon, kidney and pancreas. These results indicated that β-Pix regulated NHE3 through a PDZ domain-based adaptor, shank2 and that it had a significant role in the fine regulation of transepithelial salt and water transport.

1711/B172

WNK4 Regulates M3R Activity via Reduced Surface Expression.

W. Chung, J. Kim, K. Kim; Department of Pharmacology and Brain Korea 21 Project for Medical Science Yonsei University College of Medicine, Seoul, South Korea

With-No-Lysine(K) kinase 4 (WNK4) is known to act as a molecular switch in ion transporter insertion into the membrane. Previous studies show that WNK4 kinase regulates various ion transporters such as NCC, NKCC, and ROMK. In the present study, we found that WNK4 regulates not only ion channel but also a G-protein coupled receptor (GPCR), Muscarinic receptor 3 (M3R). This study investigates a novel regulatory mechanism of WNK4 on the surface expression of M3R. Immunoprecipitation(IP) assay of M3R was performed with WT and Q562E WNK4 mutant in HEK293 cells. WNK4 interacted with the non-glycosylated M3R and this interaction was decreased in Q562E WNK4 mutant. M3R consists of 7 transmembrane-spanning with 3 hydrophilic intracellular and 3 hydrophilic extracellular loops. Previous studies show that the third intracellular loop of M3R mediates numerous protein-protein interactions. We further investigated whether the third intracellular loop mediated M3R and WNK4 interaction, IP was performed with full length and truncated M3R constructs in HEK293 cells. This data shows that M3R and WNK4 interacted through the third intracellular loop of M3R. Using cell surface biotinylation assay, we compared cell surface expression of M3R upon co-expression of WT or Q562E WNK4 mutant. WNK4 decreased the cell surface expresssion of glycosylated M3R and this effect was partially reversed by Q562E mutant. Immunostaining of M3R revealed that WNK4 inhibited M3R surface expression by accumulating M3R in the Golgi. To investigate the effect of WNK4 on the activity of M3R, Pilocarpine-induced Ca2+ evocation by M3R was measured using Fura-2. HEK293 cells were transiently transfected with M3R and mock vector or WNK4. Pilocarpine, a muscarinic receptor agonists, was used to activate M3R. Consistently, it showed that WNK4 decreased the pilocarpine-induced Ca2+ evocation by M3R. These results indicate that activity of Muscarinic receptor 3 was regulated by WNK4 via reduced surface expression.

Exocytosis: Regulated Secretion (1712 – 1727)

1712/B173

Development of a Full-Length FRET Epac2 Sensor and Its Application.

M. Katoh, C. Zhang, T. Shibasaki, S. Seino; Division of Cellular and Molecular Medicine, Kobe University Graduate School of Medicine, Kobe, Japan

Epac (also referred to as cAMP-GEF) is a guanine-nucleotide-exchange factor (GEF) for the Ras-like small GTPases Rap1 and Rap2 that is activated by direct binding of cAMP, and two isoforms, Epac1 (cAMP-GEFI) and Epac2 (cAMP-GEFII), have been identified. We recently found that activation of Epac2-mediated signaling regulates insulin granule excytosis in a protein kinase A (PKA)-independent mechanism. Although partial and full-length Epac1 sandwiched between cyan and yellow fluorescent proteins have been developed as FRET (fluorescence resonance energy transfer) sensors to monitor Epac1 activation in living cells, a full-length Epac2 FRET sensor has not yet been reported. In this study, we have developed a FRET-based dynamic function analysis system using CFP-full-length Epac2-YFP fusion construct (termed C-Epac2-Y). We found that 8-Bromo-cAMP, a cAMP analog, decreased the FRET response in C-Epac2-Y-expressing COS-1 cells in a dose-dependent manner. We then screened for effects of various insulin secretagogues on change in FRET in mammalian cells with C-Epac2-Y. Interestingly, we found that tolbutamide and glibenclamide, sulfonylureas widely used in the treatment of diabetes, significantly decreased the FRET response. In addition, we found that insulin secretion stimulated by these sulfonylureas was significantly reduced in the pancreatic islets of mice in which Epac2 is disrupted specifically in pancreatic beta cells. These results suggest that Epac2 mediates sulfonylurea-induced insulin secretion. The present study also shows that Epac2 has diverse effects in cell signaling. Development of a full-length Epac2 FRET sensor should be useful in investigation of the mechanisms of sulfonylurea-induced signaling as well as cAMP-induced signaling.

1713/B174

HSP40:CSP Crosstalk: Implication for Neuroprotective Mechanisms at the Level of the Synapse.

S. J. Gibbs[1], B. Barren[2], N. O. Artemyev[2], J. Braun[1]; [1]Physiology and Biophysics, University of Calgary, Calgary, AB, Canada, [2]Molecular Physiology and Biophysics, University of Iowa, Iowa, IA

In neurons, stress-induced and constitutively expressed molecular chaperones protect against damage induced by ischemia and neurodegenerative diseases, however the molecular basis of this protection is not known. Here we have investigated the crosstalk between stress-induced chaperones and cysteine string protein (CSPα). CSPα is a constitutively expressed synaptic vesicle protein bearing a J domain

and a cysteine rich "string" region that has been implicated in the long term functional integrity of synaptic transmission and the defense against neurodegeneration. In this report we demonstrate that in response to heat shock or treatment with the Hsp90 inhibitor, geldanamycin, the J protein, Hsp40, becomes a major component of the CSPα complex. Association of Hsp40 with CSPα decreases CSPα-CSPα dimerization and enhances the CSPα-induced increase in steady state GTP hydrolysis of Gαs. In view of the crucial importance of stress-induced chaperones in the protection against cell death, our data attribute a role for Hsp40 crosstalk with CSPα in neuroprotection.

1714/B175

Phorbol-12-Myristate-13-Acetate Facilitates Exocytosis and Vesicle Fusion via Protein Kinase C Alpha in PC12 Cells.

R. Xue, Y. Zhao, P. Chen; Division of Bioengineering, Nanyang Technological University, Singapore, Singapore

Phorbol-12-myristate-13-acetate (PMA), which is a stable analogue of an important signaling membrane lipid diacylglycerol (DAG), has been shown to significantly potentiate exocytosis and modulate vesicle fusion kinetics in neurons and endocrine cells. The exact mechanisms underlie PMA influences, however, often remain unclear largely because of the diversity of DAG/PMA receptors that involve in the exocytotic process including, most notably, various protein kinase C (PKC) isoforms. In the present study, by overexpressing wild-type PKCalpha (wt-PKCalpha) or dominant negative PKCalpha (dn-PKCalpha), we investigated the roles of PKCalpha, which is the most abundant conventional PKC expressed in secretory cells and whose activation depends on both DAG/PMA and Ca2+, in PMA mediated regulations on exocytosis. Using amperometry measurements based on carbon fiber microelectrodes, we demonstrate that, in PC12 cells, PMA increases the extent of exocytosis, facilitates fusion pore expansion, and enlarges quantal release essentially through activation of PKCα.

1715/B176

Differential Regulation of Neutrophil Exocytosis by the Rab27a Effectors JFC1/Slp1 and Munc13-4.

J. L. Johnson[1], A. A. Brzezinska[1], D. Munafo[1], K. Crozat[2], B. Beutler[2], W. B. Kiosses[3], B. A. Ellis[1], S. D. Catz[1]; [1]Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, CA, [2]Genetics, The Scripps Research Institute, La Jolla, CA, [3]Core Microscopy Facility, The Scripps Research Institute, La Jolla, CA

Neutrophils play a central role in the innate immune response. They contain granular molecules that contribute to the execution of all neutrophil functions. The molecular mechanism that regulates exocytosis in neutrophils remains elusive. Recently, we presented evidence that Rab27a regulates azurophilic granule exocytosis in vivo. However, the mechanism underlying this regulation and whether Rab27a and its effectors regulate exocytosis of other organelles in neutrophils remain unknown. Here we show that murine neutrophils deficient in Rab27a fail to secrete myeloperoxidase (MPO) from azurophilic granules. By electron microscopy, we confirmed the subcellular localization of Rab27a on azurophilic granules. Rab27a was also detected in structures that resemble gelatinase (MMP-9) granules. Using antibody-mediated molecular interference (AMMI) we show that Rab27a regulates secretion of both azurophilic and gelatinase granules in neutrophils. Contrarily, interference with the Rab27a effector JFC1 inhibited MPO but not MMP-9 secretion. A role for JFC1 in MPO secretion was confirmed using JFC1-downregulated granulocytes which showed deficient MPO secretion. Using TIRFM we found that JFC1-downregulated cells have a significant increase in the number of moving vesicles and in the number of granules undergoing fast movement, a type of movement previously related to microtubule-associated trafficking. These data suggest that JFC1 might play a role in vesicle transition from cytoskeleton-associated movement to docking thus decreasing the number of moving vesicles and the number of vesicles undergoing fast movement. Our immunofluorescence analysis indicated that the Rab27-effector Munc13-4 is also expressed in human and murine neutrophils. Using Jinx, a mouse model of Munc13-4-deficiency, we found decreased secretion of MPO and mobilization of IL10r and LAMP-2 in Munc13-4-null neutrophils. Electron microscopy analysis showed no gross granular abnormalities in Jinx granulocytes. Using AMMI we found that MMP-9 secretion is also deficient when interfering with Munc13-4 function. We conclude that while JFC1 regulates MPO secretion, Munc13-4 appears to play a more general role in the regulation of neutrophil exocytosis.

1716/B177

Rab11a Regulates Exocytosis of Discoidal/Fusiform Vesicles in Bladder Umbrella Cells.

P. khandelwal[1], W. G. Ruiz[2], E. Balestreire[3], O. A. Weisz[4], J. Goldenring[5], G. Apodaca[6]; [1]Medicine, University of Pittsburgh, Pittsburgh, PA, [2]Medicine, University of Pittsburgh, Pittsburgh, PA, [3]Medicine, University of Pittsburgh, Pittsburgh, PA, [4]Medicine, University of Pittsburgh, Pittsburgh, PA, [5]Surgery and Cell and Developmental Biology, Vanderbilt University, Nashville, TN, [6]Medicine, University of Pittsburgh, Pittsburgh, PA

The discoidal/fusiform vesicles (DFV) of bladder umbrella cells undergo regulated exocytosis in response to filling, but little is known about their biogenesis or the molecular machinery that modulates this process. We observed that Rab11a was expressed in umbrella cells (but not Rab11b or Rab25) and was associated with DFV. Using adenovirus-mediated delivery we transducted umbrella cells in situ with either dominant active (DA) or dominant negative (DN) mutants forms of Rab11a. The over expression of DA-Rab11a stimulated an increase in apical surface area in the absence of stretch, while DN-Rab11a inhibited stretch-induced changes. Endocytosed fluid and membrane markers had little access to Rab11a-positive DFV, but virally expressed human growth hormone (hGH), a secretory protein, was packaged into DFV. While expression of DA-Rab11a stimulated release of hGH into the bladder lumen, expression of DN-Rab11a had the opposite effect. Our results indicate that DFV may be biosynthetic in nature and that their exocytosis depends on the activity of the Rab11a GTPase.

1717/B178

βPix -Activated Rac1 Stimulates the Phospholipase D Associated with Exocytosis in Neuroendocrine Cells.

F. Momboisse, E. Lonchamp, V. Calco, M. Ceridono, N. Vitale, M. F. Bader, S. Gasman; INCI CNRS, Strasbourg, France

Exocytosis is a ubiquitous and fundamental process governing many cellular physiological reactions including hormone and neurotransmitter release, and cell migration, membrane repair and proliferation. This process requires coordinated interactions between actin and the secretory granules undergoing recruitment, docking and fusion with the plasma membrane. In neuroendocrine cells, the two Rho GTPases, Cdc42 and RhoA, influence the exocytotic machinery, by spatially and temporally coordinating actin dynamics and the trafic of secretory granules to the

plasma membrane. Experiments based on the use of mutant overexpression or toxins, also suggest that Rac1 might also play a role in secretion, but its functional role and the upstream and downstream events signaling pathway remained unexplored. The aim of the present study was to investigate how Rac1 controls exocytosis in neuroendocrine cells. Using PC12 cells as a secretory cell model and an RNA interference (RNAi) strategy, we demonstrate that activation of Rac1 is an essentiel step in the exocytotic process. One possible effector pathway by which Rac1 regulates exocytosis, involves Phospholipase D1, an enzyme which produces phosphatidic acid at the plasma membrane during a late step of exocytosis. Silencing of Rac1 by RNAi was found to prevent secretagogue-evoked phospholipase D1 (PLD1) activation and blocks phosphatidic acid formation at the plasma membrane. Next we focused our attention on the upstream pathway which permits Rac1 activation. Expression of a GEF dead form and silencing experiments indicated that βPix is the guanine nucleotide-exchange factor integrating Rac1 activation to the exocytotic process. Futhermore, using mutant or silencing forms of the scaffolding protein Scrib on secretion, Rac and PLD activation assay, we show that Scrib is essential for βPix/Rac1-mediated PLD1 activation and exocytosis. Altogether, our results demonstrate for the first time that secretagogue-evoked stimulation induces a sequential ordering of β-Pix, Rac1 and phospholipase D at the plasma membrane, thereby providing lipid modifications that permit to perform the exocytotic machinery more efficiently.

1718/B179

Parafusin and Its Role in Secretion and Exocytosis.

B. Satir, L. Liu; Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

Several glycolytic enzymes and their isoforms (isomorphs) have been found to be important in physiological processes other than ATP production (glycolysis). The involvement of parafusin, a member of the phosphoglucomutase superfamily with no phosphoglucomutase activity, as a critical component in the secretory pathway is demonstrated using RNA interference of parafusin (PFUS). This protein was first described and cloned from the eukaryotic microorganism Paramecium tetraurelia, but is widely found. Earlier work showed that parafusin has unusual post-translational modifications (phosphoglucosylation) coupled to stages in the exocytic process. Using RNAi, (http://paramecium.cgm.cnrs-gif.fr/RNAi). we unequivocally demonstrate that when parafusin synthesis is reversibly blocked, although cell growth is unaffected, secretory vesicles are not synthesized and packaged properly and exocytosis becomes inhibited. Parafusin clearly is a critical component of the secretory vesicle scaffold formation and for exocytosis with a different functional role from the original phosphoglucomutase, indicating that during evolution, minor nucleotide changes and/or post-translational modifications can evolutionarily add new functional dimensions to an old enzyme.

1719/B180

Myosin 2 Promotes Exocytic Fusion Pore Opening.

P. Bhat[1,2], P. Thorn[1]; [1]School of Biomedical Sciences, The University of Queensland, St Lucia, QLD, Australia, [2]Diamantina Institute, Woolloongabba, QLD, Australia

During regulated exocytosis, granules fuse with the plasma membrane forming an aqueous fusion pore through which granule content is lost. Although implicated in controlling the kinetics, and sometimes the composition, of granule content release, the mechanisms of fusion pore regulation remain unclear. Recently, we found that actin at least partially regulates this process as inhibition with latrunculin B promoted fusion pore closure (Larina et al. 2007). We now examine the involvement of the actin motor protein myosin-2 in this process. We use extracellular dye entry into the granule to indicate fusion pore opening, and probe for pore closure by the addition of a second dye after stimulation. In control conditions, agonist stimulation promotes the fusion of granules, 83% of which still had open fusion pores 5 minutes post-stimulation. After pre-treatment with the myosin-2 inhibitor, blebbistatin, 58% of granules showed closed fusion pores compared to 21% with its inactive enantiomer, suggesting a role for myosin-2 in maintaining an open fusion pore. We examined the regulatory pathway for myosin-2 using a Rho-kinase inhibitor, Y27632, and a MLCK inhibitor, ML-9. Under the above conditions, ML-9 treatment resulted in 40% closed granules, while Rho-kinase inhibition had no effect. Western blots of cell lysates showed reduced phosphomyosin expression when cells were treated with blebbistatin and ML-9, but not Y27632. These data indicate that MLCK-dependent phosphorylation of myosin-2 promotes fusion pore opening. We examined real-time fusion pore dynamics using live-cell multiphoton microscopy with an extracellular dye to repetitively photobleach identified exocytic granules and examine fluorescence recovery. Failure of fluorescence recovery after bleaching indicates a closed fusion pore. Treatment of cells with blebbistatin markedly reduced the duration of fusion pore opening in these cells from 10 min to less than 5 min ($p<0.01$), supporting the role of myosin-2 in maintaining an open fusion pore. In conclusion, this study shows that myosin-2 phosphorylation is necessary to keep the fusion pore open, indicating an important new regulatory mechanism of fusion pore dynamics.

1720/B181

Role of Phosphatidylinositol-4-Phosphate 5-Kinase Iα in Insulin Secreting B-Cells.

J. Zhang, H. Wu, R. Luo, G. Li; National University Medical Institutes, National University of Singapore, NUS, Singapore

Insulin secretion from pancreatic β-cells is tightly controlled by glucose and other regulators via a complex signaling network. It has been demonstrated that the small G-proteins Rac1 and CDC42 are involved in this scenario, though the detailed underlying cascade remains poorly understood. This study aimed to examine the possible role of phosphatidylinositol-4-phosphate 5-kinase Iα (PIP5K-Iα), a downstream effector of the G-proteins. PIP5K-Iα is a key enzyme for biosynthesis of phosphatidylinositol-4, 5-bisphosphate (PIP2) which is implicated in many cellular events. We were able to markedly knock down PIP5K-Iα in insulin-secreting INS-1 β-cells by reverse transfection of siRNA duplexes targeting the kinase, resulting in a reduction of its mRNA level by ~70% (qPCR) and protein mass (immunoblotting) by 75% at 24-96 h. Observations under confocal microscopy revealed that PIP5K-Iα knockdown led to disruption of F-actin structure (FITC-phalloidin staining) and morphological changes (rounding up of cells). PIP2 content in the plasma membrane was decreased and glucose stimulation-induced PIP2 breakdown was abolished in cells after PIP5K-Iα knockdown, assessed by transfection of a construct expressing a GFP-tagged PIP2-binding domain of PLC-γ. In addition, basal insulin secretion was doubled and total insulin release under various stimulating conditions was elevated in cells of PIP5K-Iα knockdown. When expressed as percentage increase over the basal secretion, however, insulin secretion

evoked by high (16.7 mM) glucose alone or plus forskolin (a cAMP raising agent) was significantly reduced by ~50%, whereas high K+-induced insulin release was not affected in these cells. Moreover, PIP5K-Iα knockdown enhanced the basal metabolic rate but reduced metabolism at high glucose as determined by MTS tetrazolium assay. The cells of PIP5K-Iα knockdown also exhibited more depolarized resting membrane potential. Taken together, our results suggest that PIP5K-Iα may function not only as a downstream effector to mediate Rac1-induced organization of F-actin structure and regulation of insulin secretion, but also possibly play a role in the proximal step of signaling transduction for insulin secretion in β-cells.

1721/B182

Insulin-Stimulated Glucagon-Like Peptide-1 Secretion from the Intestinal Endocrine L Cell Promotes the Activation and Association of Cdc42 and PAK1.

G. E. Lim[1], J. Sun[2], T. Jin[1,2,3], P. L. Brubaker[1,3]; [1]Physiology, University of Toronto, Toronto, ON, Canada, [2]Laboratory Medicine and Pathobiology, University of Toronto, Toronto, ON, Canada, [3]Medicine, University of Toronto, Toronto, ON, Canada

Glucagon-like peptide-1 (GLP-1) is secreted from the intestinal L cell in response to nutrient ingestion. The primary function of this intestinal hormone is anti-diabetic, increasing both glucose-dependent insulin secretion and satiety, and decreasing glucagon release and gastric emptying. Previously, we have demonstrated that insulin induces GLP-1 secretion from GLUTag cells, a murine L cell model, through a MEK-ERK1/2-dependent mechanism. Furthermore, insulin treatment induced a transient depolymerization of filamentous actin, peaking 15 min after treatment. As in other endocrine cells, depolymerization of filamentous actin with latrunculin B was found to potentiate insulin-induced GLP-1 secretion, while stabilization with jasplakinolide inhibited GLP-1 release. Rho GTPases and their downstream kinases, such as Cdc42 and PAK1, respectively, are also required for hormone secretion from endocrine cells, and we have now determined their roles in insulin-induced GLP-1 release. GLUTag cells were transfected with an empty vector control or plasmids expressing dominant-negative constructs of Cdc42(T17N). As determined by radioimmunoassay, overexpression of Cdc42(T17N) completely abrogated insulin-stimulated GLP-1 secretion, but did not affect PMA-induced GLP-1 release. Similar results were observed when siRNAs were used to reduce Cdc42 expression, In other cell types, PAK1 is an established effector kinase of Cdc42, and binding of Cdc42 to PAK1 relieves the auto-inhibition of PAK1. Co-immunoprecipitation studies were thus performed to measure the association of PAK1 with Cdc42 in GLUTag cells. Under basal conditions, association of PAK1 with Cdc42 was not detectable by immunoblotting but, 20 min after insulin treatment, a strong association of Cdc42 with PAK1 was observed. Activation of PAK1 was also demonstrated, by immunoblotting for PAK1 phosphorylation at Thr423, with a maximal increase detected at the 20 min time point. In summary, we demonstrate for the first time a role for the Rho GTPase, Cdc42, in regulating insulin-induced GLP-1 secretion from the intestinal L cell. Furthermore, we have identified PAK1 as a potential effector kinase that is responsible for Cdc42-regulated GLP-1 release from the L cell.

1722/B183

A Role for Ubiquitination in the Insulin-Regulated Trafficking of GLUT4.

R. McCann[1], J. Stöckli[2], C. Lamb[1], D. James[2], N. Bryant[1]; [1]University of Glasgow, Glasgow, United Kingdom, [2]Diabetes and Obesity Programme, Garvan Institute of Medical Research, Sydney, QLD, Australia

Insulin regulates whole body glucose homeostasis by acting on its target tissues. A major consequence of insulin binding to its receptor on fat and muscle cells is mobilisation of the facilitative glucose transporter GLUT4 from an intracellular store to the cell surface where it serves to clear glucose from the bloodstream. The signal for sorting GLUT4 into this intracellular store is, at present, unknown. We have demonstrated evolutionary conservation between the insulin-regulated trafficking of GLUT4 and the nitrogen-regulated trafficking of the general amino acid permease Gap1p in the yeast Saccharomyces cerevisiae, and taken advantage of this finding to reveal this signal. Gap1p is retained in the endosomal system when yeast are grown on nitrogen-rich media, but traffics to the plasma membrane under low nitrogen conditions. This regulated trafficking is controlled by ubiquitination of the transporter. We have expressed human GLUT4 in yeast and demonstrated that it traffics in the same nitrogen-regulated manner as Gap1p, dependent upon ubiquitination. We find that GLUT4 is ubiquitinated in both yeast and adipocytes, and that a ubiquitin-resistant version fails to translocate to the cell surface of adipocytes in response to insulin. Our data identify ubiquitination as a signal for the trafficking of GLUT4 from the endosomal/TGN system into its intracellular storage compartment from where it can be mobilised to the cell surface in response to insulin.

1723/B184

Molecular Mechanisms of Secretory Granule Recycling in Neuroendocrine Cells: Role of Scramblase-Mediated Dynamics of Phosphatidylserine.

M. Ceridono, F. Momboisse, S. Ory, V. Calco, A. Haeberle, Y. Bailly, M. Bader, S. Gasman; Neurotransmission and Neuroendocrine Secretion, CNRS UMR 7168-INCI, Strasbourg, France

In neuroendocrine cells, release of hormones and neuropeptides occurs through a tightly calcium-regulated exocytotic process of large dense-core secretory granules. To allow secretory vesicle recycling and to maintain a constant cell surface area, exocytosis must be followed by compensatory membrane uptake. Most of our knowledge regarding secretory granules trafficking is related to stages preceding granule fusion with the plasma membrane. How these cells, specialized for hormone release, initiate and regulate compensatory endocytosis remains poorly understood. The aim of the present work was to investigate the molecular mechanisms governing compensatory endocytosis of the secretory granule membrane during neuroendocrine secretion. By following internalization of antibodies against dopamine-beta-hydroxylase (a secretory granule marker) in cultured chromaffin cells, we show here that the granule membrane is selectively recaptured after exocytosis by a clathrin-dependent process. Granule fusion with the plasma membrane is accompanied by calcium-dependent scramblase activation in the granule membrane resulting in the translocation of phosphatidylserine (PS) from the cytoplasmic lipid leaflet to the luminal leaflet transiently exposed at the cell surface. Expression of a scramblase mutant that inhibits outward translocation of PS does not affect secretion but specifically inhibits the recapture of the granule membrane. Our results support the idea that scramblase-mediated lipid reorganization is one of the mechanisms that triggers and/or regulates granule membrane recapture in secreting neuroendocrine cells. This work was supported by

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1724/B185

PICK1 and ICA69 Define Three Maturation Stages of Insulin Granules and Their Deficiency Leads to Impaired Glucose Tolerance.

M. Cao[1], C. Kam[1], C. Shen[1], X. Cao[1], K. Lee[2], J. Xia[1]; [1]Department of Biochemistry, The Hong Kong University of Science and Technology, Hong Kong, China, [2]Lee Hysan Clinical Research Laboratories,Chinese University of Hong Kong, Hong Kong, China

Diabetes is a metabolic disorder characterized by hyperglycemia. Insulin, a peptide hormone synthesized and secreted by pancreatic beta cells, is the key regulator of blood glucose. The molecular mechanism governing the budding, trafficking and secretion of insulin is not fully understood. PICK1 (protein interacts with C-kinase 1) is a peripheral membrane protein with both a PDZ (PSD-95/Dlg/ZO1) domain and a BAR (Bin/Amphiphysin/Rvs) domain. ICA69 (Islet Cell Autoantigen 69 kD), an autoantigen initially identified from type-1 diabetes patients, is also a BAR domain protein-containing protein. BAR domains are banana-shaped dimers that bind to lipid membranes and initiate vesicle formation by sensing membrane curvature or actively bending membranes. We found that PICK1 and ICA69 form heteromeric BAR domain complex in pancreatic beta cells and regulate insulin trafficking. Based on their association with PICK1 and ICA69, insulin granules can be classified into three maturation stages. Furthermore, deficiencies of PICK1 and ICA69 in mice lead to glucose intolerance and other symptoms relevant to diabetes.

1725/B186

Regulation of Beta Cell Function by Phosphatidylinositol-4,5-Bisphosphate (PIP2).

A. Tomas[1], B. Yermen[1], R. Regazzi[2], J. E. Pessin[3], P. A. Halban[1]; [1]Department of Genetic Medicine and Development, University of Geneva Medical School, Geneva, Switzerland, [2]Department of Cell Biology and Morphology, University of Lausanne, Lausanne, Switzerland, [3]Departments of Medicine and Molecular Pharmacology, Albert Einstein College of Medicine, Bronx, NY

PIP_2 is a critical second messenger that regulates diverse cellular activities such as vesicle traffic, actin remodelling and apoptosis. Although PIP_2 has been involved in regulated exocytosis, its role has not been clearly defined. Objective: To examine the role of PIP_2 in beta cell survival and insulin secretion. Methods: The well-differentiated transformed mouse pancreatic beta cell line MIN6B1 was used. TUNEL and caspase-3 activity experiments were performed to examine cell survival. Human growth hormone secretion (used as a surrogate marker for insulin secretion from transfected cells) was analysed by ELISA. Cells were examined by immunofluorescence + confocal microscopy. Results: PIP_2 was localised to actin and gelsolin-rich domains at the plasma membrane. Blocking PIP_2 action with PH-PLC-GFP or with phosphatidylinositol 4-phosphate 5-kinase I (PIP5KI) RNAi did not reduce the secretory response to glucose. However, both treatments resulted in increased sensitivity to induced apoptosis. Over-expression of PIP5KI resulted in a marked inhibition of insulin secretion and accumulation of intracellular vacuoles coated with actin and PIP_2. These vacuoles, which have also been observed in PIP5KI over-expressing adipocytes and in cells expressing constitutively active Arf6 Q67L, did not contain insulin but were positive for the endosomal marker caveolin 1, Arf6 and the insulin granule membrane protein phogrin. Expression of Arf6 Q67L also resulted in the formation of the vacuoles and in a similar pattern of inhibition of regulated secretion, which was improved by blockage of PIP_2 action. On the other hand, inhibition of the RhoA/ROCK signaling pathway improved stimulated secretion of cells over-expressing PIP5KI without affecting vacuole formation. Constitutively active RhoA V14 resulted in increased cortical actin polymerisation which was also reduced by blockage of PIP_2 action. Conclusions: PIP_2 plays a pro-survival role in MIN6B1 cells. However, excess PIP_2 produced by PIP5KI inhibited regulated secretion accompanied by a halt in the endocytic recycling of secretory membrane (in the case of Arf6-activated PIP5KI) or by the perturbation of actin cytoskeleton remodelling (due to RhoA/ROCK activation of PIP5KI).

1726/B187

Ca2+/Calmodulin-Dependent Phosphorylation of Tumor Protein D52 May Be Mediated by CAMK2δ6.

C. Chew, X. Chen, H. Zhang, H. Zhang; IMMAG, Medical College of GA, Augusta, GA

Tumor protein D52 (CSPP28, CRHSP28) is highly expressed in cells that undergo classical exocytosis, as pancreatic acinar, gastric chief and intestinal goblet cells, and is thought to play a regulatory role in this process. Cholinergic stimulation or elevation of $[Ca^{2+}]i$, but not PKC activation, increase the phosphorylation of D52 on serine residues. Using preparative 2D gel electrophoresis coupled with mass spectrometry, we identified a single in vivo Ca^{2+}-dependent phosphorylation site, S^{136}, and raised a phosphospecific antibody against this site. The goal of this study was to use the antibody as a tool to isolate and identify the protein kinase(s) that regulate S^{136} phosphorylation in vivo. Normal gastric cells were used as models along with T84 colon cancer cells and human embryonic kidney (HEK) 293 cells expressing HA-tagged D52. Combined in vitro and cellular approaches identified a protein kinase with an apparent Mr of 46 kDa. Although relatively resistant to inhibition by the Ca^{2+}/calmodulin-dependent protein kinase 2 (CAMK2) antagonist, KN93, this kinase possessed all of the classical characteristics of CAMK2 in that it bound to calmodulin Sepharose 4B, was inhibited by W7, cross-reacted with several pan-specific CAMK2 antibodies and, upon activation with Ca^{2+}/calmodulin, cross-reacted with anti-active CAMK2 ($pT^{286/287}$) antibody. Based on extensive analyses of structures and expression patterns on known CAMK2 isoforms, we hypothesized that the 46 kDa kinase was CAMK2δ6, which is structurally similar to the brain CAMK2α isoform. Expression of CAMK2δ6 in mouse gastric cells was confirmed by RT-PCR and co-expression of EGFP-tagged D52 and HA-tagged CAMK2δ6 in HEK cells demonstrated a similar pattern of subcellular distribution. These data provide the first evidence to support a role for CAMK2δ6 in modulating D52 functions that have been linked to vesicular trafficking events surrounding exocytosis. (Supported by NIH R01 DK 31900).

1727/B188

Novel Proteolysis of SNARE Proteins.

P. Fletcher[1], M. Fletcher[1], K. Weninger[2], T. E. Anderson[2], B. M. Martin[3]; [1]Microbiology and Immunology, Brody School of Medicine, Greenville, NC, [2]Physics, North Carolina State University, Raleigh, NC, [3]Laboratory of Neurotoxicology, National Institute of Mental Health, NIH, Bethesda, MD

Tissue treated with scorpion venom and selected purified components demonstrates their capabilities to enter cytoplasm and selectively cleave essential SNARE protein components. Polyacrylamide gel electrophoresis-based immunoblots of homogenates from tissues used in these assays revealed significant alterations that are characteristic of this venom and some of its purified fractions. From these studies we learned of the presence in this venom of a metalloprotease that cleaves SNARE family proteins. Initial determinations found unique cleavage of VAMP-2 and VAMP-8, the v-SNARE proteins associated with vesicle membranes. Our continuing studies have now detailed two cleavage sites in the t-SNARE SNAP-25. Related experiments in brain similarly reveal cleavage of the cognate SNARE proteins, synaptobrevin and SNAP-25. The cleavage sites are immediately adjacent to the coiled-coil domain in assembled SNARE complexes, yet cleavage appears absent in this tetrameric form. Immunocytochemical determinations reveal that SNARE proteins are cleaved in vitro in intact pancreatic lobules by proteolytic activity associated with clinically relevant venom treatment. Cloned substrate proteins that were expressed as truncated cytoplasmic portions provided cleavage site identification.

Endocytosis I (1728 – 1747A)

1728/B189

Role of Hrb in Clathrin Dependent Endocytosis.

T. Galli, M. Chaineau, L. Danglot, V. Proux-Gillardeaux, A. van der Linden; INSERM Avenir Team, Dpt Cell Biology/Institut Jacques Monod, UMR7592 CNRS & Univ. P7, Paris, France

HIV Rev binding protein (Hrb), also called human Rev Interacting Protein (hRIP) or Rev/Rex Activation domain-Binding (RAB) is a partner of the tyrosine kinase substrate Eps15 and it has been recovered in the AP2 interactome. Eps15 and AP2 are involved in endocytosis, but the function of Hrb in this process is still unknown. Here, we have identified Hrb as a partner of the vesicular SNARE Tetanus neurotoxin Insensitive Vesicle-Associated Membrane Protein (TI-VAMP, also called VAMP7) in yeast two hybrid screens and using biochemical assays. TI-VAMP is a v-SNARE that mediates an exocytic pathway which is crucial for neuritogenesis. Furthermore, TI-VAMP regulates the exocytosis of post-Golgi vesicles and is important for several specialized cellular functions linked to cell surface expansion in non-neuronal cells including phagocytosis, cell migration and invasion, and mitosis. In HeLa cells, Hrb localizes both in the nucleus and in the cytoplasm. In the cytoplasm, Hrb colocalizes with clathrin, AP2, Eps15 and transferrin receptor containing vesicles. Hrb and TI-VAMP do not significantly colocalize in control conditions in HeLa and MDCK cells but treatment with Hepatocyte Growth Factor (HGF or scattering factor) induces the colocalization of TI-VAMP and Hrb in MDCK cells. Using a TI-VAMP-GFP construct, we find that Hrb and TI-VAMP colocalize close to the plasma membrane after 5 minutes of anti-GFP antibody uptake, suggesting that TI-VAMP and Hrb may interact only during the early stages of endocytosis. Furthermore, uptake experiments followed by fluorescence-activated cell sorting (FACS) show that the endocytosis of flurorescent transferrin is strongly reduced in Hrb knock-down cells. Altogether, these results suggest that Hrb is involved in clathrin-dependent endocytosis and may recruit TI-VAMP in this process.

1729/B190

Clathrin Heavy Chain Isoforms Mediate Distinct Functions in Endosomal Sorting.

C. Esk, C. Chen, F. M. Brodsky; GW Hooper Foundation, University of California, San Francisco, San Francisco, CA

Clathrin heavy chain 17 (CHC17) is the main component of the well defined clathrin vesicle coat required for several membrane transport routes in the cell. Also, CHC17 has been implicated in Golgi organization and mitotic spindle formation. In humans a second isoform of clathrin heavy chain, CHC22, exists. Despite 85% sequence identity between the two clathrin heavy chains it is not clear if they share cellular functions. In this study, we compare the roles of clathrin heavy chains in HeLa. CHC22 was localized on intracellular membranes partly overlapping with CHC17 and markers of late endosomes. Unlike CHC17 CHC22 is not recruited onto mitotic spindles and depletion of CHC22 does not affect endocytosis, establishing these functions as unique to CHC17. However, CHC22 is increasingly recruited onto membranes in the absence of CHC17 and both clathrin heavy chains are involved in sorting various cargoes in the endosomal system. CHC22-function was localized in retrograde endosome-to-TGN trafficking, downstream of both CHC17- and retromer- mediated sorting of mannose-6-phosphate receptor and shiga-toxin at the early endosome. Both CHC17- and CHC22-function contribute to Golgi morphogenesis as Golgi formation was more profoundly affected in the absence of both clathrin heavy chains when compared to depletion of CHC17 alone. Similar distinction in clathrin heavy chain function was observed in differentiated skeletal muscle cultures, the tissue exhibiting highest expression of CHC22. This data establishes the endosome-to-TGN transport as the pathway in which CHC22 exerts its function in skeletal muscle.

1730/B191

Uptake and Trafficking of Fluorescently Conjugated Dextran and Transferrin: a Comparison of Salivary Gland Fibroblasts in Live Rodents and Isolated Fibroblasts in Culture.

A. Masedunskas[1,2], R. Weigert[1]; [1]NIDCR, National Institutes of Health, Bethesda, MD, [2]Biology, University of North Carolina, Chapel Hill, NC

Endocytosis and membrane trafficking have been studied mainly in two-dimensional cell cultures. Although these systems provide a valuable tool to investigate molecular machineries, they might not represent the characteristics of the cells in their native tissues. Recently, we described an experimental system based on intravital two-photon microscopy for studying endocytosis in live animals. We have shown that systemically injected molecules such as fluorescently conjugated dextran and bovine serum albumin diffuse out of the vasculature and are rapidly internalized by the fibroblasts residing in the stroma of the salivary glands. Indeed, we showed that the intravital microscopy can be used as a powerful tool to study the dynamics and mechanisms of endocytosis in live animals. In this study we further characterized the uptake and trafficking of the fluid-phase markers and the receptor mediated endocytosis by examining the internalization of fluorescently labeled dextran and transferrin *In Vivo*. Surprisingly, we observed the early endosomes containing labeled dextran as early as 2-5 minutes after injection, however, transferrin internalization and accumulation was markedly slower and appeared only 10-15 minutes after probe

injection. Furthermore, a significant fraction of transferrin-positive endosomes overlapped with dextran labeled endosomes. In contrast, the isolated salivary gland fibroblasts exhibited very rapid kinetics of transferrin internalization and showed no significant overlap with dextran-labeled endosomal-lysosomal system, as previously reported. Moreover, dextran internalization was markedly slowed down and mostly relied on macropinocytosis in cultured fibroblasts. In summary, our study has provided new insights into the dynamics of fluid phase and receptor mediated endocytosis in live animal, which might enable us to better understand the mechanisms of endocytosis in cells in their native environments.

1731/B192

New Trafficking Routes for Cargo Proteins Entering Cells via Clathrin-Independent Endocytosis.

C. A. Eyster, R. Huebner, J. Higginson, N. Porat-Shliom, R. Weigert, J. G. Donaldson; NHLBI, NIH, Bethesda, MD

Resident plasma membrane proteins can be endocytosed into cells by several different mechanisms, including Clathrin-Dependent Endocytosis (CDE) and Clathrin-Independent Endocytosis (CIE). While much is known about the machinery and trafficking signals necessary for CDE, less is known about the mechanisms underlying CIE. Our lab has focused on studying CIE by following the trafficking of the major histocompatibility complex Class I protein (MHC1) in HeLa cells. MHCI enters cells via CIE and traffics to the "classical" transferring-containing early endosome, where it is sorted either towards lysosomal degradation or for recycling back to the plasma membrane. MHC1 returns to the plasma membrane in distinct tubular recycling endosomes that lack transferrin. The small GTPase, Arf6, is important for controlling CIE. Expression of a constitutively active form of Arf6 (Arf6Q67L) leads to the generation of vacuolar structures that trap CIE cargo immediately after endocytosis, blocking the convergence with transferrin-containing early endosomes. In this study, we isolated these Arf6Q67L induced CIE vacuoles and analyzed their protein composition. We identified and confirmed that a variety of new endogenous cargo proteins (CD44, CD55, CD98, CD147, GLUT1 and ICAM1) are internalized into cells by CIE. Interestingly, a subset of these new CIE cargo molecules, CD44, CD98 and CD147, do not follow the same intracellular trafficking pattern as MHC1, GLUT1, and ICAM1. CD44, CD98, and CD147 appear to bypass all transferrin-containing compartments and recycle directly back to the plasma membrane in CIE recycling tubules. A divergent itinerary for this subset of newly identified CIE cargos suggests a possible sorting step in the CIE pathway. The identification of new endogenous plasma membrane proteins that are internalized into cells by CIE provides a useful set of potential cargo proteins to probe CIE in different cell types.

1732/B193

Endocytic Hot Spots Are Spatially Organized by the Actin Cortex and Actin Binding Proteins.

D. Nunez[1,2], M. Stober[1], M. Mettlen[1], S. Schmid[1], G. Danuser[1], D. Loerke[1]; [1]Cell Biology, The Scripps Research Institute, La Jolla, CA, [2]Bioengineering, University of California, San Diego, La Jolla, CA

Clathrin-mediated endocytosis (CME) is the main mechanism by which cells internalize molecules from the extracellular space. As such, it is involved in all functions regulating cellular homeostasis. Whether CME sites are spatially organized and coordinated remains a highly debated issue. While some work has suggested that CME tends to reoccur at sites of preferred nucleation termed 'hot spots', data has suggested that these same sites are the result of random nucleations restricted spatially by areas of no nucleation on the cell membrane. The goal of this research is to resolve whether CME is spatially organized, and to determine the mechanistic role of the molecules regulating this organization. Using Total Internal Reflection Fluorescent Microscopy (TIRF) in conjunction with computational image analysis of the spatial dynamics of clathrin-coated structures (CSSs), we show that ~70% of CSSs nucleate at preferred sites rather than being randomly distributed. We present an algorithm that determines the location of these sites and establishes exact time tables of initiations, abortions, internalizations, and re-initializations of CSSs in hot spots. We investigated the diffusion of CCSs within these hot spots and found confinement radii in agreement with the membrane matrix model. (Fujiwara et al.,J Cell Biol. 2002 Jun 10;157(6):1071-81.) We further tested the requirements for formation of hot spots and found an actin dependence. We therefore tested the role of proteins that link actin to CME in the formation of these hot-spots. Our data suggest that the actin cortex serves as a scaffold which creates chemical micro-environments defined by individual mesh sections that contain the endocytic proteins necessary to preferentially nucleate CCSs.

1733/B194

Pak1 and Pak2 Control Clathrin-Caveolae-Independent Endocytosis by Regulating Cortactin Association with N-WASP.

A. Grassart, A. Dautry-Varsat, N. Sauvonnet; Biologie Cellulaire et Infection, Institut Pasteur, Paris, France

Receptor-mediated endocytosis is an essential process for cells since it controls many functions including nutrient and growth factor uptake, hormone responses, antigen presentation and pathogens entry. Several endocytic routes exist, dependent or independent on clathrin. This study focuses on the interleukin 2 β receptor (IL-2Rβ) uptake that is clathrin- and caveolae-independent but dynamin- and actin- dependent. We are currently characterizing its regulation in comparison with the clathrin-dependent pathway. For the first time, we identified two specific regulators for this clathrin-caveolae independent pathway: Rac1 and p21-activated kinases (Paks). Moreover, our data revealed that cortactin, an actin polymerization regulator, is required for IL-2Rβ endocytosis and is a downstream target of Paks, suggesting a control of its function. In particular, we observed that Pak1 and Pak2 can phosphorylate cortactin on serine residues 405 and 418. Consistently, we observed a specific inhibition of IL-2Rβ endocytosis when the cells overexpressed a cortactin mutated for these residues. In addition, we identified the actin polymerization enhancer, N-WASP, as a novel essential actor of IL-2Rβ endocytosis. Strikingly, we observed that phosphorylation of cortactin by Paks increased the binding affinity of cortactin to N-WASP. Therefore, we propose a model on the specific regulation of clathrin-caveolae-independent endocytosis by Pak1 and Pak2. The induction of the Paks will lead to the phosphorylation of cortactin that will enhance its binding to N-WASP and hence would promote actin polymerization during IL-2Rβ endocytosis.

1734/B195

Identification of Two Distinct Retrograde Transport Pathways from the Early Endosome to the Trans-Golgi Network.

Z. Lieu, M. Derby, P. Gleeson; Biochemistry and Molecular Biology and Bio21 Molecular Science and Biotechnology Insitute, Unversity of Melbourne, Melbourne, VIC, Australia

Retrograde transport pathways from early/recycling endosomes to the Golgi apparatus are critical for the recycling of a range of membrane proteins, as well as the internalisation of bacterial and plant toxins. However the individual pathways remain poorly characterised. Here we have defined two distinct retrograde transport pathways from the early endosomes to the TGN that differentially transport TGN38 and Shiga toxin. Experimental evidence for the two retrograde transport pathways is summarised as follows. (1) By tracking the transport routes of the two cargos in HeLa cells we have demonstrated that TGN38 is transported directly from early endosomes to the TGN, whereas Shiga toxin trafficks to the TGN via the recycling endosomes. (2) Exit of both cargoes from the early endosome requires the Vps26-Vps29-Vps35 trimer of retromer, whereas sorting nexin 1 (SNX1) is specifically required for transport of Shiga toxin while SNX2 is required for transport of TGN38, indicating roles for these two sorting nexins in distinct transport pathways. (3) Transport of the two cargoes requires different TGN components. The TGN golgins, GCC88 and GCC185, independently regulate the trafficking of TGN38 and Shiga toxin, respectively [1,2]. Also, the TGN t-SNARE, syntaxin 6, is required for the retrograde transport of TGN38, but not Shiga toxin, to the Golgi apparatus. (5) Transport of both cargos requires syntaxin 16. In syntaxin 16 depleted cells, TGN38 accumulates in the early endosome whereas Shiga toxin accumulates in the recycling endosome, confirming the distinct pathways of each cargo. Overall, these findings have identified two retrograde transport pathways from the early endosome to the TGN and have identified components that specifically regulate each of these pathways 1. Derby, MC, Lieu, ZZ, Brown, D, Stow, JL, Goud, B, & Gleeson, PA (2007) Traffic 8, 758. 2. Lieu, ZZ., Derby, MC, Teasdale, RD, Hart, C, Gunn, P, & Gleeson, PA (2007) Mol Biol Cell. 18, 4979

1735/B196

The Recycling Endosome Is Critical for Retrograde Toxin Traffic.

D. Sheff, J. McKenzie; Pharmacology, University of Iowa College of Medicine, Iowa City, IA

The recycling endosome is a critical transit point for recycling membrane, surface receptors and membrane proteins undergoing transcytosis. More recently it has also been implicated in delivery of basolateral secretory traffic. We have been investigating the mechanism by which retrograde traffic from the early endosomes is delivered to the Golgi. Using Shiga toxin B subunit (STxB) and Cholera toxin B subunit (CTxB) as markers of the retrograde pathway, we find that both toxins transit the recycling endosome as they pass from early endosomes to the trans-Golgi network. Ablation of the recycling endosome with HRP-transferrin and DAB completely blocks access of these toxins to the Golgi. Ablation was specific for the recycling endosome as it did not prevent transferrin recycling out of early endosomes. Together, these results suggest that retrograde traffic of CTxB and STxB must pass through the recycling endosome. To further define the mechanism of passage through the recycling endosome, we examined the role of EHD proteins in toxin traffic, finding that while EHD-1 is required for transferrin exit from the recycling endosome to the plasma membrane, it is not required for the retrograde route. In contrast, both recycling and retrograde routes were sensitive to aluminum fluoride and wortmannin treatments suggesting the involvement of G proteins and PI3-Kinases in both pathways. We have further defined passage through the recycling endosome using soluble mutants of syntaxins required for retrograde traffic of endogenous proteins. STxB remains trapped in the recycling endosome when soluble syntaxin 6 or 16 is expressed. Because syntaxin 6 is involved in retrograde traffic of TGN38/46 through the recycling endosome, these results strongly support a model in which retrograde toxin traffic passes from early endosomes to the recycling endosome to the TGN. They also suggest that direct early endosome to TGN and late endosome to TGN routes are not employed by these toxins.

1736/B197

Amphiphysin: a Novel Regulator of Endocytic Recycling.

S. Pant[1], M. Sharma[3], K. Patel[1], S. Caplan[3], C. Carr[2], B. Grant[1]; [1]Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ, [2]Department of Pathology and Laboratory Medicine, UMDNJ, Piscataway, NJ, [3]Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE

Receptor mediated endocytosis is crucial for the internalization of receptor-bound ligands of many types. Cargo laden internalized vesicles fuse with early endosomes. Endosome maturation allows sorting with degradation in the late endosomes and lysosomes or recycling to the plasma membrane to participate in further rounds of endocytosis. Endocytic recycling maintains cellular homeostasis of membrane and lipid components and in polarized cells it allows accurate sorting to maintain distinct apical and basolateral domains. We have previously established that the RME-1 protein is found on the endocytic recycling compartment and mediates endocytic recycling in several *C.elegans* tissues. RME-1 is a conserved protein homologous to four mammalian proteins EHD1-EHD4. The protein is an ATPase with an ATP binding N-terminal P-loop that is critical for membrane association and homo-oligomerization. The recent EHD2 crystal structure reveals that, while being ATPases, EHD/RME-1 family proteins bear structural similarity to the clathrin coated pit GTPase Dynamin. The C-termini of RME-1 family proteins contain a single Eps15-homology (EH) domain. Classically, EH domains are found in endocytosis proteins functioning as protein interaction motifs, binding target proteins through Asparagine-Proline-Phenylalanine (NPF) motifs. Mammalian EHD proteins can bind to interacting proteins such as Syndapin, Rabenosyn5, EHBP1 and Numb in this manner. Utilizing an RNAi based approach in a GFP-RME-1 expressing worm strain, we screened for proteins causing alteration in recycling endosome morphology. We focused on predicted worm proteins bearing multiple NPF motifs. Through this screen we identified *C. elegans* AMPH-1/Amphiphysin as a novel regulator of endocytic recycling. We utilized genetic analysis to establish a novel role for AMPH-1 in the regulation of recycling endosome morphology and recycling of transmembrane cargo. We demonstrate by orthogonal approaches that RME-1 colocalizes with and associates with AMPH-1 and the association has a role in recycling. We propose a model for AMPH-1~RME-1 function at the recycling endosome bearing similarity to the mammalian Amphiphysin ~ Dynamin functional partnership at the clathrin coated pit.

1737/B198

Receptor Downregulation during Early C. Elegans Development.

J. R. Mayers[1], A. Sarkeshik[2], S. Hempel[1], J. R. Yates III[2], A. Audhya[1]; [1]Biomolecular Chemistry, University of Wisconsin-Madison, Madison, WI, [2]Dept. of Cell Biology, The Scripps Research Institute, La Jolla, CA

In *C. elegans* oocytes, a number of receptors necessary for nutrient uptake are constitutively recycled between internal endosomal compartments and the plasma membrane. However, following fertilization, several of these membrane components are no longer necessary

and are retargeted to the late endosomal system for degradation. This process is rapid and can occur within a single cell cycle (~30 minutes). Similarly, growth factor receptor signaling in mammalian cells is downregulated via receptor degradation to prevent hyperproliferation. In both cases, a set of four endosomal sorting complexes (ESCRTs) are intimately involved. However, the mechanisms that underlie the rapid change in membrane fate remain unclear. To address this problem and explore the necessity of the ESCRT complexes in early embryogenesis, we have initially taken a biochemical approach. Using antibodies generated against *C. elegans* Tsg101, an ESCRT-I subunit, we immunoprecipitated a group of interacting proteins from embryo extracts. These included two known components of the complex, Vps28 and Vps37, as well as a recently identified fourth subunit, Mvb12. Additionally, using polycistronic coexpression and purification from bacterial lysates, we found that Mvb12 behaves as a stoichiometric subunit of the complex. Several other proteins that were not previously known to interact with ESCRT-I were also identified by mass spectrometry, which are currently under investigation. These new proteins may play important roles in the reorganization of membrane trafficking that occurs after developmental cues such as fertilization.

1738/B199

EHBP-1 Functions with RAB-10 during Endocytic Recycling in C. Elegans.

A. Shi, C. Chen, R. Banerjee, B. Grant; Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ

Recently our lab showed that RAB-10, the *C. elegans* ortholog of mammalian Rab10, functions in endocytic recycling, mediating transport from basolateral early endosomes to basolateral recycling endosomes in intestinal epithelia. A similar role was found for Rab10 in MDCK cells, suggesting a conserved mechanism regulates this pathway in metezoa. In a yeast two-hybrid screen for binding partners of RAB-10(Q68L) we identified Ce-EHBP-1, and confirmed the interaction biochemically using GST-pulldowns. Ce-EHBP-1 is a homolog of human Ehbp1, a calponin homology domain (CH) protein previously shown to interact with EHD1 and EHD2 during endocytic transport of GLUT4 in mammalian adipocytes. Interestingly Rab10 was also recently shown to regulate insulin stimulated GLUT4 transport in adipocytes. In *C. elegans* we find that EHBP-1-GFP, driven by the *ehbp-1* promoter, is widely expressed in many tissues. EHBP-1 colocalizes with RAB-10 on early endosomal structures in the intestine suggesting that these proteins function together on this organelle. In EHBP-1 knockdown and knockout animals, recycling cargo hTAC-GFP accumulates in internal membranes, and RAB-5-positive early endosomes accumulate to abnormally high levels, while recycling endosome number is greatly reduced. All of these phenotypes mimic those observed in rab-10 mutant animals, further indicating the functional link between RAB-10 and EHBP-1. Surprisingly, loss of EHBP-1 interferes with endosome association of RAB-10, but loss of RAB-10 does not disrupt EHBP-1's endosomal association, confounding the typical pattern of RAB-GTPase and effector. The role of the actin-cytoskeleton in endocytic transport remains poorly understood, especially with regard to its function on intracellular membranes such as endosomes. Several proteins that regulate the actin cytoskeleton have been implicated in endosome function, including Arf6, Cdc42, Myosin Type V, and Syndapin/Pacsin. The CH domain of EHBP1, and the observed association of mammalian Ehbp1 with actin filaments in vivo, suggests an intriguing link between RAB-10 and actin during receptor recycling.

1739/B200

Computational Dissection of Adenovirus Movements on the Cell Surface.

C. Burckhardt, P. Schönenberger, U. F. Greber; Institute of Zoology, University of Zürich, Zürich, Switzerland

The earliest steps of virus host cell interactions are incompletely understood. In the case of many human adenoviruses they involve primary virus attachment to the coxsackie virus B adenovirus receptor CAR. Secondary interactions with alpha v integrins are required for clathrin and dynamin mediated endocytic uptake, which leads to infection. CAR is a type one transmembrane protein involved in cell-cell adhesions on the basolateral domain and in tight junctions. It is unknown how the virus switches from CAR to integrins and engages in endocytosis. Here we are using live fluorescence imaging at high temporal resolution in combination with single particle tracking algorithms to systematically map the movements of adenoviruses on human epithelial cells. We find that individual virus trajectories are of considerable heterogeneity. To unravel the information contained within these trajectories, we developed a novel trajectory segmentation approach based on supervised support vector classifications. This approach revealed three distinct patterns, diffusion, drifts and confined motions on filopodia and the cell body. Upon attachment, viruses were mostly randomly diffusive, before they processively drifted or were confined. Adenovirus drifts and infection required filamentous actin and myosin 2. The absence of CAR reduced Ad2 drifts whereas alpha v integrin depletion increased the drifts, suggesting a competition between CAR and integrins in viral surface motions. Fluorescence recovery after photobleaching of GFP-CAR confirmed the high surface motility of CAR. Our data suggest a model where CAR binding allows Ad2 to scan the plasma membrane while alpha v integrin engagement traps the virus at sites that can lead to endocytosis. This work shows how a combination of live fluorescence imaging and computational analyses can reveal new features of the plasma membrane organization, which are important for infectious virus uptake.

1740/B201

Sorting Nexin 5: a Role in the Biogenesis of Macropinosomes.

J. T. Wang[1], M. C. Kerr[1], N. Hamilton[1], J. P. Lim[2], P. Gleeson[2], R. Teasdale[1]; [1]Institute of Molecular Biosciences, The University of Queensland, Brisbane, QLD, Australia, [2]The Department of Biochemistry and Molecular Biology, Bio21 Molecular Science and Biotechnology Institute, The University of Melbourne, Melbourne, VIC, Australia

Macropinocytosis is a high capacity variant of endocytosis which is initiated at sites of dynamic actin-mediated plasma membrane reorganization. Despite being implicated in the immune response, modulation of cell signaling, as well as host-pathogen manipulations, the molecular machinery involved in macropinocytosis is still largely undefined. In order to address the shortcomings of quantitative studies on macropinocytosis in the past, we have exploited the tractability of macropinosomes via microscopy to develop an image-based quantitative assay utilizing 3D confocal microscopy. Utilizing this assay, bone-marrow derived macrophages exhibited a 60% drop in macropinosome number after treatment with LPS, which also correlated with a 40% decrease in the level of Sorting Nexin 5 (SNX5). Moreover, in HEK-Flp-In cells stably overexpressing SNX5, the number of macropinosomes both constitutively and in response to EGF-stimulation is elevated 100% relative to control cells. Inhibition of the tyrosine kinase activity of the EGF receptor through the application of AG1478 prevented SNX5's translocation to the plasma membrane, and correspondingly prevented the formation of macropinosomes in response to EGF.

Together these results implicate that not only does SNX5 play a role in the generation of macropinosomes, its translocation to the cell surface in response to EGF treatment is also involved in macropinocytosis.

1741/B202

Sonic Hedgehog Regulates Pinocytic Membrane Trafficking during Axon Growth and Retraction.

A. Kolpak, J. Jiang, Z. Bao; Medicine, UMass Medical School, Worcester, MA

During development, axons extend through the extracellular environment and are guided to their appropriate targets by axon guidance factors. These factors can induce axon turning, growth or retraction. Both axon growth and retraction require cytoskeletal and membrane reorganization, however, the membrane trafficking events mediating the response of axons to guidance factors are poorly understood. Here, we show that axon membrane retraction is associated with the formation of clathrin-independent, dextran-positive pinocytic vesicles in the growth cone. A negative factor, a high concentration of Sonic Hedgehog (Shh), induced axon retraction and increased dextran uptake. A positive factor, a low concentration of Shh, decreased dextran uptake and additionally increased the motility and turnover of these vesicles. Disruption of F-actin dynamics and inhibition of dynamin function decreased dextran uptake and membrane retraction. These results suggest that this type of pinocytic vesicle constitutes a critical component of membrane trafficking during axon growth and retraction.

1742/B203

Differential Sorting and Fate of Neuropathy-Associated Antibodies to GM1 Ganglioside and Cholera Toxin after Internalization in Epithelial and Neuronal Cells.

R. Iglesias-Bartolomé, R. Comin, A. L. Moyano, J. L. Daniotti; Departamento de Quimica Biologica, CIQUIBIC (CONICET-UNC), Facultad de Ciencias Quimicas, Universidad Nacional de Cordoba, Cordoba, Argentina

Gangliosides are glycolipids mainly located at the plasma membrane (PM). Antibodies to gangliosides have been associated with a wide range of neuropathy syndromes. Particularly, antibodies to GM1 ganglioside are present in patients with Guillain-Barré syndrome (GBS) and in a rabbit model of this disease. In this work, we investigated in different cell types the binding and intracellular fate of affinity purified IgG and IgM antibodies to GM1 (GM1Ab) obtained from a rabbit model of GBS in comparing with the transport of cholera toxin (CTx), which binds with a high affinity GM1. We demonstrated that in GM1 expressing CHO-K1 cells, GM1Ab is rapidly and specifically endocytosed. After internalization, the antibodies transited sorting endosomes to accumulate at the endocytic recycling compartment, no colocalizing with lysosomal markers. About 50% of the endocytosed GM1Ab is recycled back to the PM and released into the culture medium. This endocytic recycling route in CHO-K1 cells was only partially followed by CTx: endocytosed GM1Ab colocalized to some extent with co-endocytosed CTx at early and recycling endosomes, but not in Golgi complex and endoplasmic reticulum, where CTx was also located. GM1Ab, in contraposition to CT, showed a reduced internalization in COS-7 cells and neuronal cell lines AH-SY5Y and Neuro2a. Results from live cell imaging and fluorescent recovery after photobleaching studies revealed an inverse correlation between the efficiency of endocytosis and lateral mobility of GM1Ab in PM, suggesting that the immobile fraction of GM1 observed in CHO-K1 cells could probably arise from domain formation, which in turn could be responsible for the increased internalization of GM1Ab observed in this cell line. Taken together, results indicate that GM1Ab and CTx are differentially endocytosed, providing the basis to gain further insight into the mechanisms that operates in the intracellular trafficking and pathological effect of CTx and neuropathy-associated antibodies.

1743/B204

Intracellular Sorting of Galectins and Their Potential Role in Protein Targeting.

M. C. Carlsson, C. Cederfur, H. Leffler; MIG, Laboratoriemedicin, Lund, Sweden

Galectins are defined by shared affinity for β-galactosides and significant sequence similarity in the carbohydrate recognition domain (CRD). Each galectin has unique carbohydrate specificity which seems to determine its biological functions, such as cell adhesion, growth and death. These effects require its carbohydrate binding ability but the underlying mechanisms are still unknown. However, there are indications that intracellular galectins can be involved in protein targeting which could explain some of their cellular effects. We have previosly shown that alterations in galectin carbohydrate binding change its intracellular distribution, suggesting an intracellular sorting task for the evolutionary conserved fine specificity. Moreover, endocytosis experiments in fibroblast cells have shown that two galectins, galectin-3 and galectin-8 use different intracellular pathways. Galectin-8 is recycled to the cell membrane by the NPC-1 protein pathway, since disruption of this path cause galectin-8 accumulation in the cytosol while galectin-3 is unaffected by this blockage. To investigate the functional role for intracellular galectins endocytosis of galectin-3 binding and non-binding transferrin (separated by affinity chromatography) was studied in HFL-1 cells. Flourecence microsopy show that galectin-3 binding transferrin uses a completely different intracellular sorting pattern compared to the non-binding transferrin. A significant amount of galectin-3 binding transferrin was recycled back to the cell surface and was completely colocalized with galectin-3 while non-binding transferrin was retained in cytosol and only to a minor degree colocalized with galectin-3. These results demonstrate that galectins could identify certain proteins based on their glycosylations and target them to different cellular compartments. This suggest a carbohydrate dependent intracellular sorting mechanism responsible for some of the galectin biological effects.

1744/B205

Microtubule Dependent Transformation of Rat Intestinal Epithelial Cells by Rab25.

L. A. Lapierre[1,2], C. M. Caldwell[1,2], K. M. Avant[1,2], R. Beauchamp[1,2,3], J. Goldenring[1,2,3]; [1]Surgery/Epithelial Biology, Vanderbilt Univ. Medical Center, Nashville, TN, [2]Nashville VA Medical Center, Nashville, TN, [3]Vanderbilt-Ingram Cancer Center, Nashville, TN

Epithelial cells regulate their cell surface compositions through selective endocytosis and recycling of membrane proteins. Little is known of how membrane recycling pathways influence transformation and neoplasia. We have previously identified the small GTPase Rab25 as an epithelial-specific modulator of membrane recycling. Recent studies have demonstrated that Rab25 expression is upregulated in a number of epithelial cancers and over-expression may increase the aggressive phenotype of subsets of breast and ovarian cancers. Recently we have utilized the non-transformed Rat Intestinal Epithelia (RIE) cell line to examine the influence of Rab25 on cell transformation. Tetracycline-

regulated overexpression of Rab25 in RIE cells leads to morphological transformation, growth in soft agar and tumor formation in nude mice. These results have led us to hypothesize that Rab25 acts as a tumor promoter with activation of specific downstream signaling cascades. In the presence of doxycycline (off) the cells form an orderly monolayer with organized cell: cell contacts. However, removal of doxycycline (on) elicits a marked alteration in cell morphology with disruption of cell contacts and adoption of a scattered morphology. Both the transformed phenotype and the growth in soft agar can be reversed by treatment with either nocodazole or indibulin, two microtubule destabilizers. This induced morphological transformation was not observed in an RIE line that inducibly overexpressed GFP-Rab11b. Interestingly these cells also exhibited spacial segregation of the Rab11 family members and their effectors. While Rab11a and Rab11-FIP1 localized adjacent to the GFP-Rab25 membranes, Rab11b and Rab11-FIP5 (Rip11/pp75) were totally separate from the GFP-Rab25. These results indicate that overexpression of GFP-Rab25 in RIE cells leads to microtubule-dependent transformation and also alters elements of the plasma membrane recycling system.

1745/B206

Caspase-8 Regulates Rab5 Function and Endosome Trafficking.

V. A. Torres[1], A. Mielgo[1], D. Anderson[2], D. Stupack[1]; [1]Department of Pathology and Moores UCSD Cancer Center, University of California San Diego, La Jolla, CA, [2]Cancer Research Unit, Health Research Division, Saskatchewan Cancer Agency, Saskatoon, SK, Canada

Caspase-8 is a cysteine protease widely characterized in the extrinsic pathway of apoptosis, which is known to be dependent on early endosomes. Previously, we identified caspase-8 as an effector of migration, promoting motility in a manner dependent upon phosphorylation on Y380 by Src family kinases, and its subsequent association with SH2 domain containing proteins. Here, we describe a novel role for caspase-8 by regulating the small GTPase Rab5, which mediates early endosome formation and trafficking. The regulation of Rab5 does not require proteolytic activity, but does require an intact phosphorylation site at Y380. Caspase-8 phosphorylation on Y380 mediates binding to p85α, a regulator subunit of PI3'Kinase with potent Rab-GAP activity via its BH domain. The binding blocks the Rab-GAP activity of p85α and promotes Rab5 GTP loading, leading to the accumulation of Rab5 positive endosomes at the edge of the cell and altered trafficking of cargo from early to late endosomes. Caspase-8 dependent GTP-loading of Rab5 is overcome by increased expression of p85α, but requires the phosphotyrosine binding activity of the SH2 domains as well as the Rab-GAP activity within the BH domain. Thus, we document a novel signaling function for phosphocaspase-8 as a regulator of p85α Rab-GAP activity and endosomal trafficking.

1746/B207

Exocytosis of Acid Sphingomyelinase upon Cell Injury Triggers Endocytosis and Plasma Membrane Repair.

C. Tam[1], V. Idone[1], C. M. Devlin[1,2], I. Tabas[2], N. Andrews[1,3]; [1]Section of Microbial Pathogenesis, Yale University School of Medicine, New Haven, CT, [2]Medicine, Columbia University, New York, NY, [3]Cell Biology, Yale University School of Medicine, New Haven, CT

Survival of eukaryotic cells after injury requires restoration of plasma membrane integrity. Earlier studies showed that plasma membrane permeabilization triggers a Ca2+-dependent repair response involving lysosomal exocytosis (1) and a rapid form of endocytosis (2). However, it was not known if lysosomal exocytosis and endocytosis were functionally linked. Here we show that lysosomal exocytosis is required for the Ca2+-dependent endocytosis observed after cell wounding. Treatment with bromoenol lactone (BEL) blocked lysosomal exocytosis, and also inhibited injury-dependent endocytosis and plasma membrane repair. We were able to specifically block injury-induced endocytosis with desipramine, an inhibitor of the lysosomal enzyme acid sphingomyelinase (ASMase). Desipramine-treated cells exocytosed lysosomes in response to Ca2+, but were defective in endocytosis and plasma membrane resealing. In humans, ASMase mutations cause the lysosomal storage diseases Niemann-Pick type A and B. Similar to what was observed after desipramine treatment, fibroblasts and lymphoblasts from Niemann-Pick Type A patients were not impaired in beta-hexosaminidase secretion, but were deficient in injury-induced endocytosis and plasma membrane resealing. Importantly, ASMase enzymatic activity was detected in the extracellular medium when cells were injured in the presence of Ca2+. Thus, Ca2+ influx through plasma membrane wounds delivers lysosomal acid sphingomyelinase to the cell surface. We propose that secreted ASMase converts plasma membrane sphingomyelin into ceramide, a sphingolipid known to induce membrane invagination and the formation of large endosomes (3). These results shed light on the mechanism by which lysosomal exocytosis triggers endocytosis, and the subsequent removal of plasma membrane lesions. (1) Reddy, A et al. Plasma membrane repair is mediated by Ca2+-regulated exocytosis of lysosomes. Cell 106, 157-169 (2001). (2) Idone, V et al. Repair of injured plasma membrane by rapid Ca2+-dependent endocytosis. J. Cell Bio 108, 905-914 (2008). (3) Zha, X et al. Sphingomyelinase treatment induces ATP-independent endocytosis. J. Cell Biol. 14), 39-47 (1998).

1747/B208

Characterizing Functional Roles of DNMBP in Endocytosis and in the Mouse Central Nervous System.

R. Bagshaw, O. Rocks, J. Jin, J. Ruston, T. Pawson; Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, ON, Canada

Rho GTPases are governed by a family of Rho GEF and GAP proteins which function in controlling the GTP-bound state of Rho proteins. Our lab is interested in how the interplay between Rho GEFs and GAPs function in establishing and maintaining cell polarity, and the possible reciprocal functionality of individual Rho GEFs and GAPs. Hypothetically, two proteins which may reciprocally function as a 'Rho GEF/GAP pair' are the Rho GEF Rich1/Nadrin and the Rho GAP DNMBP/Tuba. In common, these proteins contain BAR domains, are active towards cdc42, and have previously been shown to be functional towards aspects of tight junction formation and maintenance in epithelial cells. To further explore the function of these proteins, we have developed and begun characterization of two mouse lines harbouring 'Cre'-conditional 'floxed' gene trap insertions in DNMBP and Rich1 genes. Mice homozygous for the DMNBP gene trap insertion are viable and fertile and display almost complete ablation of DMNBP expression by western blot of brain lysate, without concomitant expression of Cre. We have also further characterized the putative role of DNMBP in early steps of endocytosis by examining the binding-partners for the SH3 domains of DNMBP, and live-cell microscopy of fluorescent-tagged DNMBP. Through this, we have found evidence of DNMBP being involved in specialized endocytic uptake rather than for general endocytic events. These findings and this work will help elucidate mechanisms of endocytosis, cargo selection, and cell-junction configuration involving these proteins and the Rho pathway.

1747A/B209

APLP2 Expression Regulates Endocytosis of MHC-Peptide Complexes through a Clathrin-Dependent Pathway.

A. Tuli[1,2], M. Sharma[1], H. Capek[2], N. Naslavsky[1], S. Caplan[1], J. C. Solheim[1,2,3]; [1]Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE, [2]Eppley Institute for Research in Cancer and Allied Diseases, University of Nebraska Medical Center, Omaha, NE, [3]Department of Pathology and Microbiology, University of Nebraska Medical Center, Omaha, NE

Immune responses against viruses and tumors rely on the presentation of viral and tumor-derived peptides to cytotoxic T-lymphocytes by cell surface receptors known as major histocompatibility complex (MHC) class I molecules. Little is known about the proteins regulating trafficking of MHC-peptide complexes to and from the cell surface. One candidate protein that we have shown to be associated preferentially with peptide-bound forms of the murine MHC class I molecule K^d is amyloid precursor-like protein 2 (APLP2). In our recent study, we have demonstrated that overexpression of APLP2 enhances endocytosis of K^d, leading to its reduced surface expression and stability. Our findings indicate that APLP2 associates with K^d both at the cell surface and in endocytic vesicles. To elucidate the mechanisms by which increased APLP2 expression causes enhanced endocytosis of K^d molecules, we analyzed how APLP2 itself undergoes endocytosis. Our findings indicate that APLP2 is internalized in a clathrin-dependent manner, as a dominant negative dynamin II mutant and the C-terminal tail of AP180 (both inhibitors of clathrin-dependent endocytosis) block its internalization. However, endocytosis of K^d is not blocked by these inhibitors, consistent with findings by others that MHC class I molecules are internalized mainly through a clathrin-independent pathway. Furthermore, the APLP2 cytoplasmic tail contains consecutive tyrosine-based NPXY and YXXØ motifs that can potentially bind to adaptor protein AP-2. Knock-down of the AP-2 μ2 subunit by siRNA or mutation of the tyrosine in the NPXYXXØ sequence leads to severely impaired internalization of APLP2, indicating the functionality of this motif in APLP2 endocytosis. Interestingly, this APLP2 Y755A mutant, unlike wild-type APLP2, fails to enhance the endocytosis of K^d molecules. Moreover, expression of dynamin II K44A also blocks the enhanced endocytosis of K^d induced by APLP2 over expression. These findings suggest a model whereby increased expression of APLP2 causes recruitment of clathrin machinery at the site of APLP2 association with K^d molecules at the cell surface, resulting in enhanced uptake of K^d in a clathrin-dependent manner.

Cilia and Flagella III (1748 – 1773)

1748/B210

Characterizing the Role of Cep72 in Cilia Formation and Function.

T. Stowe, R. Hoh, T. Stearns; Biological Sciences, Stanford University, Palo Alto, CA

Cilia are composed of a microtubule axoneme surrounded by a membrane bilayer, and are nucleated by a specialized centriole. Cilia are found on many mammalian cell types, and have been implicated in extracellular signaling, fluid movement and development. To identify genes for regulatory and structural components involved in cilium formation, we performed subtractive transcriptional microarray analysis of ciliating mouse tracheal epithelial cells. These cells generate hundreds of centrioles and cilia during differentiation in culture. One of the identified upregulated genes, Cep72, was found to colocalize with the pericentriolar satellite proteins PCM-1 and BBS4, which are implicated in a known ciliopathy, Bardet-Biedl syndrome. BBS4 is a core member of the BBSome, a conserved protein complex thought to be involved in transport of ciliary membranes and associated proteins. Interestingly, overexpression of Cep72 mislocalizes both PCM-1 and BBS4 to cytoplasmic aggregates without perturbing localization of other centrosomal proteins. In addition, overexpressed Cep72 can be coimmunoprecipitated with PCM-1. BBS4 localizes to both pericentriolar satellites and primary cilia, whereas Cep72 is confined to cytoplasmic pericentriolar satellites and is not detectable within primary cilia. We are investigating the role of Cep72 in regulation of pericentriolar satellites and primary cilium formation, and particularly its possible role in BBSome localization and function.

1749/B211

Chlamydomonas Cyclic GMP-Dependent Protein Kinase II Has a Role in Ciliogenesis.

Q. Rasi, J. Parker, G. Alfaro, C. T. Beh, L. Quarmby; Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada

We have isolated a new mutant strain of Chlamydomonas reinhardtii with defects in flagellar assembly. pkg2 mutant cells exhibit a range of flagellar length phenotypes, including cells with no visible flagella, short flagella, and flagella of unequal length. We have cloned the gene disrupted in these cells and identified it as PKG2, a member of the type II family of cyclic GMP-dependent serine/threonine protein kinases. PKG2 is one of three PKGs identified in the Chlamydomonas flagellar proteome. All three are type II PKGs and phylogenetic analysis indicates that type I PKGs are only found in animals. Localization and fractionation studies show that PKG2 is a cytosolic protein, with a small fraction appearing in the flagella. We have identified a PKG2-interacting protein using yeast two-hybrid and are testing the relevance of this protein to the flagellar assembly phenotypes. In mammals, PKGIIs are implicated in a variety of cellular functions including intestinal and renal fluid secretion, bone growth and regulation of circadian rhythm. Our results suggest that these phenotypes might derive from ciliary defects.

1750/B212

Katanin Plays an Essential Role in the Pre-Mitotic Resorption of Cilia in Chlamydomonas.

J. Parker[1], Q. Rasi[1], J. Feldman[2], W. Marshall[2], L. Quarmby[1]; [1]Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC, Canada, [2]Department of Biochemistry & Biophysics, UCSF, San Francisco, CA

The microtubule-severing protein, katanin, participates in the regulation of both cilia and cell cycle progression. Independent research groups have been working on these two aspects of katanin's activity, but the relationship between the two activities has not been studied. Using an RNAi approach in Chlamydomonas, we have discovered that severe knock-down of the katanin p60 subunit, KAT1, is lethal unless the cells carry secondary mutations which affect ciliogenesis. These data lead us to propose that katanin plays an essential role in disengagement of the cilium from the basal body/centriole prior to mitosis. In support of this idea, we observe that in wild-type Chlamydomonas cells, flagellar

remnants are detected after premitotic flagellar resorption. Depending on culture conditions, these flagellar remnants range from mostly-intact flagella to what we submit to be isolated transition zones.

1751/B213

Characterization of the Dual Specificity Protein Phosphatase, MKP1, in Chlamydomonas.

N. F. Wilson, J. A. Buchheim; Anatomy and Cell Biology, Oklahoma State University Center for Health Sciences, Tulsa, OK

Chlamydomonas actively maintains flagella at an equal and appropriate length. To date, four genes have been identified that when mutated generate cells with abnormally long flagella. The LF4 gene encodes a MAP kinase present in both cell bodies and flagella. MAP kinases require the phosphorylation of a threonine and tyrosine residue of a TXY motif within the kinase domain for enzyme activity. Previous studies revealed that LF4p was phosphorylated on the TEY motif in cell bodies but not in flagella. To identify negative regulators of LF4p enzyme activity in flagella, we examined the genome sequence for the presence of dual specificity protein phosphatases (DSPs). Using the version three release of sequence, eight DSPs were identified. Northern blot analysis revealed that MKP1 is upregulated during flagellar regeneration suggesting this phosphatase could be a candidate for a negative regulator of LF4p enzyme activity. Immunoblot analysis demonstrated that MKP1 is present in cell bodies but not flagella. Similarly, immunofluorescent microscopy localized MKP1 to two discrete spots at the apical end of the cell body. Extraction of cell bodies with 0.5% NP-40 resulted in the release of ~25% of MKP1. The remaining MKP1 was not solubilized even in the presence of 0.6 M NaCl. Taken together, these results suggest that MKP1 could be associated with basal bodies. Currently, we are employing isolation of nuclear flagellar apparatuses combined with immunofluorescent and immunogold electron microscopy to confirm the localization of MKP1 to basal bodies. In addition, we are using RNAi and MKP1 substrate traps to determine what role, if any, MKP1 plays in the regulation of flagellar length and LF4p enzyme activity. Supported by OCAST grant HR-164.

1752/B214

Sentan: a Novel Specific Component of the Apical Structure of Vertebrate Motile Cilia.

A. Kubo[1], A. Yuba-Kubo[2], S. Tsukita[3], S. Tsukita[4], M. Amagai[1]; [1]Department of Dermatology, Keio University School of Medicine, Tokyo, Japan, [2]Mitsubishi Kagaku Institute of Life Sciences, Tokyo, Japan, [3]Biological Science Laboratory, Graduate School of Frontier Biosciences, Osaka University, Suita, Japan, [4]Cell Biology, Kyoto University Faculty of Medicine, Kyoto, Japan

Human respiratory and oviductal cilia have specific apical structures characterized by a narrowed distal portion and a ciliary crown. These structures are conserved among vertebrates that have air respiration systems; however, the molecular components of these structures have not been defined, and their functions are unknown. To identify the molecular component(s) of the cilia apical structure, we screened EST libraries to identify gene(s) that are exclusively expressed in ciliated tissues, are transcriptionally upregulated during in vitro ciliogenesis, and are not expressed in testis (because sperm flagella have no such apical structures). One of the identified gene products, named sentan, was localized to the distal tip region of motile cilia. Using anti-sentan polyclonal antibodies and electron microscopy, sentan was shown to localize exclusively to the bridging structure between the cell membrane and peripheral singlet microtubules, which specifically exists in the narrowed distal portion of cilia. Exogenously expressed sentan showed affinity for the membrane protrusions, and a protein-lipid binding assay revealed that sentan bound to phosphatidylserine. These findings suggest that sentan is the first molecular component of the ciliary tip to bridge the cell membrane and peripheral singlet microtubules, making the distal portion of the cilia narrow and stiff to allow for better airway clearance or ovum transport.

1753/B215

PIP2 Regulates Axoneme Assembly and Spermatid Polarity in Drosophila Spermatogenesis.

L. Fabian[1], H. Wei[1], J. Rollins[2], G. Polevoy[1], J. Brill[1]; [1]Developmental and Stem Cell Biology, Hospital for Sick Children, Toronto, ON, Canada, [2]College of Mount Saint Vincent, Riverdale, NY

Male germ cells undergo dramatic changes in cell shape and polarity during spermatogenesis, a process essential for male fertility that is highly conserved across species. Differentiation of spermatids into mature sperm requires assembly of the flagellar axoneme and orientation of the elongated spermatids in a polar fashion. Studies in budding yeast, tissue culture cells and *Arabidopsis thaliana* suggest that the membrane lipid phosphatidylinositol 4,5-bisphosphate (PIP_2) is important for cell polarity. Research from our lab indicates that PIP_2 plays several roles in regulating sperm formation in *Drosophila melanogaster*. We showed previously that *Drosophila* male germ cells lacking PIP_2 fail to form elongated spermatids and mature sperm (Wei *et al., J. Cell Sci.,* 2008). In PIP_2-depleted spermatids, microtubule-based structures, including axonemes, are defective. Here we identify a mechanism where PIP_2 is responsible for regulating axoneme assembly and characterize a role for PIP_2 in spermatid polarity. Immunoblotting of testis extracts suggested that one of the posttranslationally modified forms of tubulin was defective in male germ cells lacking PIP_2. Consistent with this, immunofluorescence and immunoelectron microscope studies revealed that polyglycylation of axonemal tubulin is a target of PIP_2 regulation. When the amount of PIP_2 in male germ cells is reduced only slightly (low-PIP_2), flies make elongated, but not mature sperm, and these males are sterile. We found that the elongated cysts in low-PIP_2 testes show a bipolar phenotype, with sperm cells oriented in opposite directions and lacking actin cones. Several cytoskeletal and elongation complex markers relocalize to the mid-region of the elongated cysts, but the exocyst, a protein complex involved in vesicle trafficking, shows diffuse localization. These results identify the exocyst complex as a potential target of PIP_2 regulation. Overall, we propose that PIP_2 has a dual role in *Drosophila* spermatogenesis, regulating both axoneme formation and cyst polarity.

1754/B216

A Novel Subunit of the Bbsome Regulates the Acetylation of Cytoplasmic Microtubules through HDAC6.

A. Loktev[1], Q. Zhang[2], J. S. Beck[2], C. Searby[2], T. E. Scheetz[2], F. Bazan[1], D. Slusarski[3], V. C. Sheffield[2], P. K. Jackson[1], M. V. Nachury[4]; [1]Cell Regulation, Genentech, Inc., South San Francisco, CA, [2]Department of Pediatrics and Howard Hughes Medical Institute, University of Iowa, Iowa City, IA, [3]Department of Biology, University of Iowa, Iowa City, IA, [4]Department of Molecular and Cellular Physiology, Stanford University School of Medicine, Stanford, CA

Bardet-Biedl syndrome (BBS) is an obesity-retinopathy-nephropathy disorder whose etiology is linked to the primary cilium. We have recently shown that seven of the twelve known BBS proteins are present in a stable complex, the BBSome, that is proposed to promote the activation of Rab8 through its association with the Rab8 GDP/GTP exchange factor, and thus drive ciliary membrane biogenesis. We have now discovered an eighth BBSome subunit, BBIP10 (BBSome-Interacting Protein of 10 kDa). BBIP10 is well-conserved and present exclusively in the genomes of ciliated organisms. Consistent with its presence inside the BBSome, BBIP10 localizes to the primary cilium, BBIP10 is required for ciliogenesis and zebrafish embryos depleted of BBIP10 display a classic bbs phenotype. Yet, the systematic analysis of BBIP10 and BBS1 to BBS12 knockdown phenotypes in RPE cells found that BBIP10 uniquely regulates the cytoplasmic microtubule (MT) cytoskeleton. Specifically, cells depleted of BBIP10 had significantly less polymerized tubulin and completely lacked acetylated microtubules while other tubulin modifications associated with stable MTs remained unaffected. The roles of BBIP10 in MT stability and acetylation appear to be independent of one another since forced stabilization of MTs by taxol in BBIP10-depleted cells does not rescue MT acetylation. Since the bulk of BBIP10 is not bound to the BBSome and the BBIP10 knockdown phenotype is not replicated by knockdown of any BBS protein, BBIP10 is likely to regulate cytoplasmic MT acetylation independently of the BBSome. Strikingly, genetic and pharmacological inhibition of the tubulin deacetylase HDAC6 rescues MT acetylation in BBIP10-depleted cells. We also show that BBIP10 physically interacts with HDAC6, thereby suggesting a role for BBIP10 in the regulation of HDAC6 activity. In summary, we have discovered a novel subunit of the BBSome that is required for ciliogenesis, but also appears to be essential for cytoplasmic MT stability and acetylation independently of the BBSome. We propose that BBSome-bound BBIP10 may function to couple acetylation of axonemal MTs - by regulating HDAC6 activity- and ciliary membrane growth.

1755/B217

Ciliogenesis Requires the Bardet-Biedl Syndrome-Associated ARL6 Protein, a Small GTPase Present at the Golgi and within or Near the Basal Body Transitional Fibers.

C. Schroeder, M. Esmail, M. Leroux; Mol. Biol. and Biochem., Simon Fraser University, Burnaby, BC, Canada

Bardet-Biedl syndrome (BBS) is a pleiotropic disorder associated with thirteen different genes and typified by obesity, polycystic kidneys, retinal degeneration, skeletal anomalies, and sensory impairment. Current evidence suggest roles for BBS proteins in trafficking proteins to centrosomes/basal bodies and within primary cilia, which are hair-like cellular extensions that perform key sensory functions in human physiology (e.g., vision, smell and hearing), and participate in various signaling pathways during development. Using a functional genomics approach, we discovered that the *BBS3* gene encodes ARL6, an uncharacterised member of the expansive ARF/ARL small GTPase family. ARF/ARLs are generally known for their roles in membrane trafficking and in various microtubule-dependent processes. Here we show that BBS3/ARL6 is found at two distinct subcellular compartments, namely the centrosome/basal body and the Golgi apparatus. In ciliated and non-ciliated cells, BBS3 localises to a distinct site within the microtubule-based centriole, independent of the molecular motor dynein and the microtubule network. In hTERT-RPE and HeLa cells, this site is at, or in close proximity to, the transitional fibers, where the intraflagellar transport (IFT) machinery docks and a potential ciliary gate analogous to the nuclear pore. We also show that in non-ciliated cells, in addition to basal body localisation, BBS3 represents the first BBS protein found at the Golgi apparatus. Biochemical assays on the wild-type protein and mutagenic variants uncovered in patients show that BBS3 depends on its ability to bind and/or hydrolyse GTP for proper function. Furthermore, overexpression of the BBS3 T31R or Q73L dominant variants leads to significant differences in the proportion of ciliated cells and cilium length. Our findings therefore establish BBS3 as a protein required for ciliogenesis, and suggest that it may perform a critical role in trafficking/sorting at the Golgi, and functions at transitional fibers, where it may assist the docking and/or sorting of ciliary cargoes before transport into the organelle.

1756/B218

Huntingtin Associates with Centrosomes and Regulates Primary Cilia Assembly.

G. Keryer[1], J. R. Pineda[1], R. Pardo[1], A. Merdes[2], S. Humbert[1], F. SAUDOU[1]; [1]Institut Curie / UMR146 CNRS, Orsay, France, [2]Institut de Sciences et Technologies du Médicament de Toulouse, Toulouse, France

Huntingtin (htt), the protein mutated in Huntington's disease, is a positive regulator for vesicular transport. Htt associates with vesicles and facilitates the microtubule (MT)-dependent transport of vesicles. A better understanding of htt function in intracellular trafficking is crucial and might reveal new pathogenic mechanisms in disease. Using different antibodies against htt and expression of several htt-tagged constructs we show that htt localizes at the centrosome in neuronal and RPE1 cells. When investigating how htt localizes to the centrosome, we found that htt is recruited in a microtubule-dependent manner. Htt interacts with huntingtin-associated protein (HAP1) that interacts with the Pericentriolar molecule 1 (PCM1), a protein involved in the transport of several centrosome components and that participates to cilia biogenesis. We show that htt and HAP1 are closely associated with PCM1 granules/satellites. Huntingtin also binds directly to dynein and to dynamitin and indirectly affects $p150^{Glued}$ through HAP1. Strikingly, when htt is silenced by RNAi approaches, the PCM1-satellites and PCM1-associated proteins pericentrin, centrin 3 and ninein become randomly dispersed whereas centrosomal γ-tubulin is not significantly altered. Because PCM1 satellites depend on dynein-mediated transport for centrosomal recruitment, our current results are consistent with an involvment of htt in centrosomal transport mediated by dynein. As PCM1 and pericentrin have been shown to be important for cilia biogenesis, we investigated ciliogenesis in htt-depleted cells and found that primary cilia formation in G0-arrested cells was affected. We conclude that htt levels regulate the basal body function of the mother centriole. Together, we suggest that htt regulates the transport of centrosome components and cilia biogenesis revealing a new regulatory role for htt that could be dysregulated in disease.

1757/B219

The Human Ciliopathy Disease Gene OFD1 Encodes a Protein that Colocalizes with PCM-1 on Centriolar Satellites.

C. A. Lopes[1], L. Romio[2], A. Merdes[3], S. A. Feather[4], A. S. Woolf[2], A. Fry[1]; [1]Biochemistry, University of Leicester, Leicester, United Kingdom, [2]Institute of Child Health, University College London, London, United Kingdom, [3]Institut de Sciences et Technologies du Médicament de Toulouse, Centre National de la Recherche Scientifique/Pierre Fabre, Toulouse, France, [4]St James' University Hospital, Leeds, United Kingdom

Oral-facial-digital (OFD) syndromes are a group of developmental disorders that feature malformations of the face, oral cavity and digits. OFD1, the most common type, is characterized by X-linked dominant transmission and is often associated with polycystic kidney disease (PKD). Studies made on cystic kidney disease genes have revealed that their products are often expressed in ciliated cell types and localize to primary cilia, basal bodies or centrosomes. This has highlighted an unanticipated link between key proteins of the centrosome/basal body/primary cilia complex and the development of severe human disease conditions. We have previously shown that the OFD1 protein localizes to the centrosome and basal body, and we therefore speculate that OFD1 may be involved in the biogenesis, maintenance or signalling function of primary cilia. Our goal is to characterize OFD1 protein at the molecular level and ultimately to unravel the biological function and importance of OFD1 for centrosome and primary cilia function. Here, we show that OFD1 not only localizes to the centrosome and basal body, but also localizes in small aggregates in its vicinity - the centriolar satellites - and that the clustering of these small aggregates depends upon an intact microtubule network and the dynein-dynactin molecular motor. Moreover, we demonstrate that OFD1 significantly co-localizes with PCM-1 (pericentriolar material 1) at the centriolar satellites and depletion studies show that these proteins are mutually dependent for correct localization to these structures. Interestingly, these studies have also shown that depletion of OFD1 reduces the efficiency of primary cilia formation. Together, our findings suggest that OFD1 is a protein critical to the organization and function of cilia through its molecular role at the centrosome, possibly via its interaction with PCM-1.

1758/B220

Cep120 Is a Daughter Centriole-Associated Protein with Roles in Centriole and Cilia Assembly.

M. Mahjoub[1], T. Stearns[2]; [1]Dept. of Biology, Stanford University, Stanford, CA, [2]Dept. of Genetics, Stanford School of Medicine, Stanford, CA

Cilia are evolutionarily conserved, microtubule-based organelles that provide cells with diverse sensory and motility functions. Cilia are nucleated by basal bodies (differentiated centrioles) that anchor the cilia just beneath the plasma membrane. Various human diseases are caused by defects in the assembly and function of cilia, as well as the basal bodies/centrioles that nucleate this organelle. Despite their importance, little is known about the mechanism(s) of centriole duplication, centriole maturation, and how centrioles initiate the formation of cilia. We have previously established an in vitro mouse tracheal epithelial cell (MTEC) culture system to study these processes (Vladar and Stearns, 2007). MTEC differentiation occurs through defined steps resulting in the formation of hundreds of centrioles, followed by growth of a cilium from each of the centrioles. We have determined the transcriptional profile of MTEC differentiation and have identified approximately 1000 genes that are upregulated during the early stages of centriole biogenesis and cilia assembly (Hoh and Stearns, unpublished data). One such gene is Cep120, which is upregulated approximately 7-fold in this dataset. Cep120 has been shown to be a centrosomal protein required for maintaining the neural progenitor pool during neocortical development in mouse (Xie et al., 2007). We find that Cep120 localizes asymmetrically to the daughter centriole in cultured mammalian cells, and that this asymmetry is maintained during cell division. In MTEC, Cep120 localizes to newly forming centrioles during the early stages of differentiation. The C-terminal coiled-coil domain of Cep120 is both necessary and sufficient for its centriolar localization. RNAi-mediated depletion of Cep120 protein causes defects in centriole duplication and cilia assembly in cultured cells and MTEC. Biochemical analysis suggests that Cep120 forms a multimeric complex at the centrioles, the components of which we are working to identify.

1759/B221

Centriole Assembly and Orientation in Planaria.

J. C. Azimzadeh[1], A. Sanchez-Alvarado[2], W. Marshall[1]; [1]Biochemistry and Biophysics, UCSF, San Francisco, CA, [2]School of Medicine, University of Utah, Salt Lake City, UT

Arrays of cilia drive fluid flow in many organ systems, including the flow of mucus in the airway and the flow of cerebrospinal fluid in brain ventricles. Ciliary flow is generated by the coordinated beating of cilia oriented in the same direction. Beating orientation is determined by the positioning of the basal bodies, from which cilia are assembled, in the plane of the plasma membrane. We are studying this process using freshwater Planaria as a model system. Planaria are flatworms that rely on a multiciliated ventral epithelium for locomotion, allowing direct observation of the phenotypes generated by RNAi against basal body/centriole and cilia proteins. We found that all the centriole genes identified in vertebrates are found in the genome of Planaria and show high sequence similarity to their vertebrate counterparts. We targeted homologs of genes required for centriole assembly in other animal systems and found that ciliogenesis was totally inhibited, supporting the idea that these proteins are also required for centriole assembly in Planaria. We have undertaken a screen for genes affecting centriole maturation and orientation. Two genes were found to be required for orientation of the ciliary beating in the axis of the body. Interestingly, those genes are putative homologs of the Chlamydomonas VFL1 and VFL3 genes. We found that knocking down those genes induces a sidewinder phenotype, i.e. the worms are moving sideways 25° to the left. The sidewinder phenotype had already been described in a systematic RNAi gene perturbation study following depletion of SmedODF2, the homolog of the vertebrate ODF2 gene (Reddien et al., Dev. Cell, 2005, 8:635-49). In this case however, worms are moving sideways to the right. We are trying to understand how those three genes interact to allow the proper positioning of basal bodies.

1760/B222

Developmental Regulation of the Ciliary Proteome in Sea Urchin.

R. Morris, B. J. Rossetti, A. P. Rawson, A. Hussein, T. Shintaku, D. Perelman; Biology, Wheaton College, Norton, MA

The sea urchin embryo has been a model of choice for studies of cilia formation and function for a half century. In most urchin species, the onset of ciliogenesis occurs on all blastomeres coordinately just prior to embryo hatching and is regulated thereafter in a tissue-specific manner to generate cilia with different lengths and behaviors. Probing the recently completed genome of the purple sea urchin Strongylocentrotus purpuratus using the flagellar proteomes from human and from the single-celled alga Chlamydomonas reinhardtii, we have begun studying how urchin embryos regulate ciliogenesis differently in different germ layer and between developmental stages. Genes representing all ciliary protein classes including a majority of the novel flagellar-associated proteins identified in the Chlamydomonas Flagellar Proteome and human ciliome, were identified in the S. purpuratus genome by reciprocal BLAST to generate a draft sea urchin

ciliome. Early analysis of S. purpuratus transcriptome data revealed that expression levels of presumptive ciliary genes vary between stages of development in four general patterns: predominantly maternal, predominantly blastular, initially blastular, and predominantly larval. Some groups of genes whose products presumably function together, for example intraflagellar transport (IFT) genes, share similar expression patterns during development, while members of other groups, for example dynein-related proteins, exhibit dissimilar expression patterns. Gene ontology analysis within and between expression pattern groups is being used to identify groups of genes that may work in concert. Genes whose products are central to specific ciliary processes, such as kinesin-2 family members that drive anterograde IFT, are being targeted by antisense morpholino knockdown to directly test their roles inferred by expression pattern and gene ontology analysis. Thus, generation of a draft ciliary proteome in a classic model organism with readily available transcriptome data is allowing us to study how the ciliogenic program can be modulated during development to generate cilia with tissue-specific morphologies, motilities, and functions.

1761/B223

From RNAi Mutants to Protein Complexes: Using Itraq, 2D DIGE Proteomics and Y2H to Reveal Protein Interaction networks in the Trypanosome Flagellum.

S. Lacomble[1], N. Portman[1], B. Thomas[1], P. McKean[2], K. Gull[1]; [1]Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom, [2]Department of Biological Sciences, Lancaster University, Lancaster, United Kingdom

Cilia and flagella are icons of eukaryotic cell biology critical to the pathogenicity of many parasites and whose malfunction is associated with an ever-growing number of human pathologies. The structural morphology of the flagellum has been well conserved and a variety of model organisms have been used to identify a cohort of widely conserved protein components. By utilising the effective reverse genetics techniques available for the flagellated protozoan parasite Trypanosoma brucei, we have generated mutant cell lines in which RNAi is targeted against specific flagellar proteins. Following purification, the comparative proteomic techniques iTRAQ and 2D-DIGE are used to detect the loss of the target protein in the isolated flagellum. In addition to the specific protein ablated by the RNAi, this approach allows detection of any protein cohort dependent upon it for incorporation into the flagellar structure. As a proof or principle, we initially identified a proteome of 30 proteins in a specific sub-compartment of the T. brucei flagellum - the paraflagellar rod (PFR). We then generated an interaction network for these proteins using a yeast two-hybrid screen which identifies a number of hub interactor proteins. Feeding these back into the RNAi/comparative proteomic pipeline has enabled us to refine further our understanding of the sub structure of the PFR and has allowed us to produce a hierarchical map of protein consortia for this structure. Given the success of this approach, we are now applying it to specific axonemal proteins and their interacting partners.

1762/B224

The Role of Old and New Flagella in Trypanosome Morphogenesis.

J. Buisson[1,2], T. Blisnick[1,2], P. Bastin[1,2]; [1]Parasitology and Mycology, Pasteur Institute, Paris, France, [2]URA2581, CNRS, Paris, France

The trypanosome is a uniflagellated protozoan parasite responsible for trypanosomiasis in Africa. It is a valuable model for studies of the flagellum as it is easy to grow and manipulate in culture, its genome is fully sequenced, reverse genetic is accessible and flagellar genes are well conserved. The flagellum is in continuity with the basal body, emerges from the cell body through the flagellar pocket at the posterior end of the cell and is attached to the cell surface. During its cell cycle, the trypanosome goes through a phase where it possesses two flagella, the old one (mature and maintained) and the new one (in construction). Both flagella stay connected through a structure called the flagellar connector that is positionned at the distal tip of the new flagellum. This structure would impose positionning of the new flagellum that influences cell morphogenesis. Blocking the construction of the new flagellum in IFTRNAi inducible strains results in cells growing shorter and shorter flagella, with a correlation between flagellum length and cell size ; non flagellated cells being the shortest ones. These observations demonstrate that the flagellum is essential for correct cell morphogenesis. To understand whether presence of the new flagellum is sufficient to ensure normal cell morphogenesis or if it needs to be guided by the old one, we set up the following system. We used inducible mutant IFT88RNAi with and without a flagellum GFP marker, triggered RNAi resulting in formation of short cells with a bald basal body. We then stopped the induction and analysed the cells (live or upon fixation) with optical and scanning electronic microscopy . « De-induced » cells could grow a new flagellum from a pre-existing basal body, but it failed to restaure normal cell morphogenesis, leading to a very heterogenous population characterized by different cell shape and multiple flagella not attached to the cell body. These results demonstrate that the new flagellum needs to be guided by the old one for correct morphogenesis.

1763/B225

A Novel RRM Domain Protein Affects A-Tubulin and the Formation of the Motile Apparatus in Marsilea Sperm Cells.

C. M. Van Der Weele, S. M. Wolniak; CBMG, Unviversity of Maryland-College Park, College Park, MD

Spermatogenesis in the water fern *Marsilea vestita* is a rapid process that reaches completion in 11 h. The single celled microspore undergoes nine division cycles to produce 32 spermatids and 7 sterile cells. Spermatids undergo *de novo* formation of basal bodies, formation of a complex multilayered cytoskeleton, nuclear remodeling and ciliogenesis. The male gametophyte is transcriptionally silent and development relies on stored protein and the translation of stored mRNA. Previously, we demonstrated that RNA processing proteins contribute to the regulation of early stages of spermatid development. We hypothesized that other RNA regulating proteins might be involved in the formation of the motile apparatus. We isolated a novel protein (MVu620) consisting of ~250 amino acids, with a 90 amino acid RNA Recognition Motif from the RRM-1 super family. The function of this protein was assessed by RNAi, using dsRNA added to spores. In severe knockdowns, cell divisions were affected; incorrect numbers and sizes of cells were observed. In milder knockdowns, divisions occurred normally, but the cytoskeleton and ciliary apparatus failed to form. In these spermatids, basal bodies were few in number and aberrantly localized. Nuclear remodeling was initiated as DAPI staining showed condensed DNA, but the nucleus was “fluffy” and failed to elongate. In situ hybridizations showed that α-tubulin mRNA was present in spermatids, but only weak labeling was observed with antibodies directed against posttranslationally modified or unmodified α-tubulin protein. In situ hybridizations show MVu620 mRNA is present in sterile cells early in development and then becomes locally detectable and eventually abundant in spermatids at the time the motile apparatus is formed.

Our efforts focus on a direct or indirect role for MVu620 in mRNA processing or translation of α-tubulin during the initial stages in cytoskeletal assembly of the motile apparatus. Supported by NSF grant MCB-0720486 to SMW.

1764/B226

The Regulation of Basal Body Formation and Cytoskeletal Assembly in Developing Spermatids of Marsilea.

F. Deeb, S. M. Wolniak; Cell Biology and Molecular Genetics, University of Maryland, College Park, College Park, MD

The microspore of the water fern, *Marsilea vestita*, contains a single cell that undergoes nine mitotic division cycles to produce 32 spermatids and 7 sterile cells. Cytoplasmic movements of stored proteins and stored mRNAs precede the first division in the transcriptionally-quiescent gametophyte, creating zones that later become spermatogenous initials. Asymmetric divisions produce jacket cells, and then, symmetric divisions produce spermatids. The spermatids differentiate into motile gametes with condensed, coiled nuclei. We developed RNAi strategies to target mRNA degradation and arrest development, and have shown that translation is controlled both spatially and temporally. We have studied the role of spermidine in spermatid development and differentiation. RNAi and drug treatments were employed to deplete spermidine in the spores at different developmental stages. dsRNA probes were made from cDNAs encoding spermidine synthase (SPDS) and spermidine transporter (SPDT) proteins. Development in the dsRNA treated spores was arrested early, before the completion of the last round of division. dsRNA-treated spores had fewer but larger spermatogenous cells than controls. Cells were labeled with anti-centrin and anti-α-tubulin antibodies to localize basal bodies and the microtubule ribbon, respectively. In dsRNA-treated spermatogenous cells, the nuclei elongated but did not condense, basal body positioning was altered and the microtubule ribbon was in disarray. An SPDS inhibitor was added at 6h, the start of spermatid differentiation. The spermatid nuclei remained round, centrin failed to localize into basal bodies and the microtubule ribbon was completely abolished. Our results show that spermidine is essential for division and development at early stages and for nuclear condensation and spermatid morphogenesis at later stages of spermatid development. Supported by NSF grant MCB-0720486 to SMW.

1765/B227

Association of Tubby-1 with the Nuclear Flagellar Apparatus in Chlamydomonas Reinhardtii.

K. E. VanderWaal[2], S. Goldstein[2], M. A. Sanders[1], M. Porter[2]; [1]Imaging Center, University of Minnesota, St. Paul, MN, [2]Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN

Cilia and flagella are complex microtubule-based organelles that perform critical sensory and motile functions. We are using a collection of motility mutants from the biflagellate alga *Chlamydomonas* to identify components that regulate flagellar motility. In previous work, we characterized a mutation (*bop5-2*) that disrupts assembly of an IC/LC complex located at the base of the I1 inner arm dynein (Bower et al., in preparation). Transformation with wild-type *IC138* restores the missing I1 subunits and increases microtubule sliding velocities to wild-type levels. However, the motility defects are not fully rescued; both *bop5-2* and *IC138* transformed cells tend to circle and spin, with little forward progression. To better understand this phenotype, we analyzed the flagellar waveforms of *bop5-2* and *IC138* transformed cells. The amplitude of the flagellar beat is increased in the *IC138* transformants, but the waveform is more symmetrical than wild-type. These results suggested another gene affecting motility might be disrupted in *bop5-2*. We found that the *bop5-2* deletion removes >20 kb of genomic DNA, including the last two exons of the *Tubby-1* (*TBY1*) gene. Studies in mice and *C. elegans* have indicated that tubby related proteins are expressed in ciliated tissues and appear to be associated with ciliopathic mutations, but their specific functions are poorly understood. To characterize *TBY1*'s activity in *Chlamydomonas*, wild-type and tagged versions of *TBY1* were transformed into *bop5-2*. The *TBY1* transformants are more effective forward swimmers, but the tagged TBY1 protein is concentrated in the cell body, not in isolated flagella. More specifically, it localizes to a ring or disk shaped structure found above the nucleus, between the two contractile vacuoles and within the nuclear flagellar apparatus. Current studies are focused on understanding how *TBY1* might influence flagellar behavior in *Chlamydomonas*.

1766/B228

Nde1 Promotes Cell Cycle Progression through Inhibition of Ciliogenesis.

S. Kim, L. Tsiokas; Cell Biology, U of Oklahoma Health Science Center, Oklahoma City, OK

The primary cilium is a nonmotile, solitary organelle that projects from the plasma membrane of most quiescent vertebrate cells. While its primary function is to detect chemical and/or mechanical signals from the surrounding cellular environment, it has also been implicated in the regulation of the cell cycle. However, the mechanisms by which cilium regulates the cell cycle are unknown. Here, we describe a new role of the mitotic protein Nde1 (or NudE) in linking ciliogenesis and cell cycle progression. Endogenous Nde1 protein expression was highest in mitosis (M) and lowest in G1/Go, inversely correlating with ciliogenesis in NIH3T3 cells. Consistently, Nde1 overexpression resulted in loss of primary cilia, while RNAi of Nde1 resulted in longer cilia and multiple cilia in a single cell. The inhibitory effect of Nde1 on ciliogenesis was rescued by co-expression with the dynein light chain (LC8). LC8 is a known interacting partner of Nde1 and a component of the dynein-mediated transport within the cilium. Overexpression of Nde1 mutants lacking association with known interacting partners, but not with LC8, led to loss of cilia. Given that Nde1 was specifically localized to the basal body, these data suggested that Nde1 inhibited ciliogenesis by limiting the amount of LC8 to the primary cilium. Next, we wanted to test whether Nde1 regulated cell cycle progression and whether this effect was cilium-dependent. Epistasis experiments in NIH3T3 cells showed that Nde1 was upstream of IFT88, a protein required for ciliogenesis, in a pathway that regulated transition from G1/G0 to S phase. Because Nde1 was also upstream of IFT88 in the ciliogenic pathway, we propose that the negative effect of Nde1 on ciliogenesis was absolutely required for normal G1/G0 to S transition. Cells lacking Nde1 would have abnormally longer cilia and thus, would require longer time to disassemble their cilia and re-enter the cell cycle compared to wild type cells with normal size cilia. This concept is consistent with the cellular basis of microcephaly, a pathological condition reported in Nde1 knock out mice, which is characterized by reduced proliferation of neuronal precursors and smaller size brain.

1767/B229

EB1 and EB3, but Not EB2, Are Required for Assembly of Primary Cilia in Fibroblasts.

J. M. Schrøder, L. Pedersen; Department of Biology, Section of Cell and Developmental Biology, University of Copenhagen, Copenhagen, Denmark

Microtubule (MT) plus-end tracking proteins (+TIPs) are a large group of structurally unrelated proteins that accumulate at the growing end of MTs. They are involved in regulating MT dynamics and in linking the MT plus end to other cellular components and organelles. End-binding (EB) family proteins are a subgroup of +TIPs that interact with a number of other +TIPs and play a major role in the targeting/tethering of these to the plus end. EB proteins also localize to centrosomes/basal bodies, and we have previously shown that centrosomal EB1 is required for assembly of primary cilia in mouse fibroblasts (Schrøder et al. 2007, Curr. Biol. 17:1134-1139); the role of other EB family members (EB2 and EB3) in ciliogenesis is unknown. We depleted EB1, EB2, or EB3 from Human Foreskin Fibroblasts (HFF) using siRNA, and analyzed the effect on primary cilia formation in growth-arrested cells using immunofluorescence microscopy. Consistent with our previous results we found that depletion of EB1 inhibits ciliogenesis by ca. 50% in growth-arrested fibroblasts (n=159). Interestingly, we found that depletion of EB3 had a similar inhibitory effect on ciliogenesis in growth-arrested HFF cells (n=156), whereas no significant effect of EB2 depletion on ciliogenesis was observed (n=153). Thus EB1 and EB3 appear to function redundantly in promoting ciliogenesis in fibroblasts, while EB2 appears to be completely dispensable for this process. We are currently using dominant-negative constructs to confirm the role of EB1 and EB3 in ciliogenesis and are also trying to determine the underlying mechanism. Supported by grants from the Danish Natural Science Research Council (272-05-0411) and The Novo Nordisk Foundation to LBP as well as a PhD fellowship from the Faculty of Science, University of Copenhagen to JMS.

1768/B230

Role of the RNA-Binding Protein Bicaudal-C during Development and Kidney Morphogenesis.

C. Maisonneuve, I. Guilleret, D. Constam; ISREC/EPFL, Epalinges, Switzerland

The aim of this project is to determine how morphogenetic signals of the TGFb and Wnt protein families might be regulated by the RNA-binding protein Bicaudal-C during kidney morphogenesis and early embryogenesis. In Drosophila, Bicaudal-C is essential during anterior-posterior patterning to localize the posterior determinant Oskar by recruiting the CCR4-NOT deadenylase to target mRNAs. In the mouse, two independent mutations in a single Bicc homolog have been identified in jcpk and bpk mice which show no early developmental defects, but instead die of polycystic kidney disease (PKD). In humans, PKD is a leading cause of renal failure attributed to impaired cilia signaling and the resulting hyperproliferation and loss of planar polarity in renal tubular epithelial cells. How cilia establish planar cell polarity is not well understood, but may involve crosstalk with non-canonical Wnt signaling. In Xenopus, Bic-C can also potentiate the TGFbeta-related activity of Nodal during gastrulation. Here, we explore whether Bicc1 interacts with Nodal in the mouse embryo, and we investigate possible mechanisms by which Bicc1 controls kidney morphogenesis to suppress cyst formation.

1769/B231

Planar Cell Polarity Proteins Act at the Level of Vesicle Trafficking to Drive Cilia Biogenesis.

J. B. Wallingford; Mol. Cell Dev. Biol., University of Texas at Austin, Austin, TX

The Wnt/planar cell polarity (PCP) signaling system governs a variety of polarized cellular behaviors that are essential for embryonic morphogenesis. We have found that in vertebrate embryos, disruption of the PCP effector genes results in embryonic phenotypes consistent with a failure of not only PCP signaling, but also Hedgehog signaling. We traced such Hedgehog phenotypes to a requirement for PCP components in ciliogenesis. Here, we will describe new roles for PCP proteins during ciliogenesis and membrane trafficking using a new in vivo model. We show that the PCP signaling components, Dishevelled (Dvl), Inturned and Fuzzy work in concert with the Rho GTPase to direct the assembly of an actin network that positions basal bodies at the apical plasma membrane. Moreover, we find that basal body docking to the apical surface involves a Dvl-dependent association of basal bodies with membrane-bound vesicles and with the vesicle-trafficking protein, Sec8. In addition, computational and in vivo experimental studies demonstrate a fundamental role in vesicle trafficking for the novel protein encoded by the PCP effector gene Fuzzy. Indeed, disruption of Fuzzy function results in inappropriate membrane fusion events, and Fuzzy interacts physically and functionally with a previously-undescribed Rab-like GTPase. Finally, Fuzzy mutant mice display embryonic defects stemming from failures of both PCP and Hedgehog signaling, but also display defects in bone growth that may be attributable to disrupted secretion. Together, our data reveal direct links between PCP proteins and fundamental components of the vesicle trafficking machinery. We propose that the control of vesicle trafficking is a core cellular function of the PCP signaling apparatus.

1770/B232

The Exocyst Protein Sec10 Is Necessary for Primary Ciliogenesis and Cystogenesis.

X. Zuo[1], W. Guo[2], J. Lipschutz[3]; [1]Medicine and Biology, University of Pennsylvania, Philadelphia, PA, [2]Biology, University of Pennsylvania, Philadelphia, PA, [3]Medicine, University of Pennsylvania, Philadelphia, PA

Primary cilia are found on many epithelial cell types, including renal tubular epithelial cells, where they are felt to participate in flow sensing and have been linked to the pathogenesis of cystic renal disorders such as autosomal dominant polycystic kidney disease (ADPKD). We previously localized the exocyst, an eight protein complex involved in membrane trafficking, to the primary cilium of MDCK cells and showed that it was involved in cystogenesis. Here, using shRNA to knockdown exocyst expression and stable transfection to induce exocyst overexpression, we show that the exocyst protein Sec10 specifically regulates renal primary cilia biogenesis. Using scanning and transmission electron microscopy, primary cilia containing only basal bodies are seen in the Sec10 knockdown cells and increased ciliogenesis is seen in Sec10 overexpressing cells. These phenotypes do not appear to be due to major changes in cell polarity, as apical, basolateral, and tight junction proteins remain properly localized. Sec10 knockdown prevents normal cyst morphogenesis when the cells are grown in a collagen matrix, whereas Sec10 overexpression results in increased cystogenesis. Transfection with human Sec10 resistant to the canine shRNA rescues the phenotype, demonstrating specificity. Finally, Par3 was recently shown to regulate primary cilia biogenesis. Par3 and the exocyst co-localized by immunofluorescence (IF) and co-immunoprecipitated. Par3 levels were unchanged in Sec10 knockdown cells; however, Par3 no longer localized to the primary cilia, consistent with a role for the exocyst in primary cilia biogenesis and the delivery of Par3 or an associated protein.

1771/B233

C. Elegans Clathrin Adaptor Protein Complex 1 (AP-1) and Cilia Assembly.

O. I. Kaplan, O. Blacque; University College Dublin, Dublin, Ireland

Cilia biogenesis and maintenance is dependent on intraflagellar transport (IFT), a bidirectional motility of multi-subunit protein complexes along the ciliary axoneme. Driven by kinesin 2 and IFT-dynein motors, IFT is thought to deliver structural and functional ciliary components to discrete parts of the organelle. Post-golgi trafficking of ciliary proteins to the base of cilia is also important for cilia assembly. To date a number of proteins have been linked to this upstream trafficking process, including RAB-8, FAPP2, BBSome components and AP-1. In *C. elegans*, AP-1 is required for targeting membrane proteins to cilia and for building intact amphid and phasmid channel cilia. Here, the role of *C. elegans* AP-1 in cilia assembly was further investigated. First, we find that all examined cilia subtypes in *unc-101* mutants (AP-1μ subunit) possess severely affected morphologies, including truncated axonemes and loss of cilia architecture. Similarly, RNAi knockdown of *aps-1* (AP-1σ subunit) in ciliated cells also disrupts cilia structure, indicating that AP-1 functions as a complex to mediate *C. elegans* cilia assembly. To determine the site of AP-1 function, we found that a rescuing UNC-101::GFP protein localizes to the trans-golgi network (TGN) region and not to cilia, and that moving UNC-101::GFP-associated particles are sometimes observed in the cell soma. Next, using *In Vivo* IFT motility assays, we found that all examined IFT proteins localise and move normally within the dendrites and cilia of *unc-101* animals, indicating that the severe ciliary defects of *unc-101* mutants are not caused by gross abnormalities in IFT. Finally, we provide evidence that loss of UNC-101 function disrupts sensory signalling pathways linked to the maintenance of cilia shape/architecture. Taken together with findings from other groups (Dwyer et al. 2001; Mukhopadhyay et al. 2008), the available data suggest that the role of UNC-101 in cilia structure determination is not served by directly regulating IFT, but rather by targeting signalling proteins, required for cilia morphology determination, from the TGN to the ciliary organelle.

1772/B234

Localization and Function of Nephrocystin-4 in Chlamydomonas.

J. Awata[1], S. Takada[1,4], K. Lechtreck[1], E. Johnson[1], B. L. Walker[2], G. Pazour[2], R. Bloodgood[3], G. Witman[1]; [1]Cell Biology, UMASS Medical School, Worcester, MA, [2]Program in Molecular Medicine, UMASS Medical School, Worcester, MA, [3]Cell Biology, University of Virginia, Charlottesville, VA, [4]Washington University School of Medicine, St. Louis, MO

Mutations in human *NPHP4*, encoding the protein nephrocystin-4, cause nephronophthisis and retinitis pigmentosa. It has been reported that the *Caenorhabditis elegans* homologues of nephrocystin-4 and the human nephrocystin-4 interacting protein, nephrocystin-1, are specifically located in the transition zone of the cilia. We observed that the *Chlamydomonas* homologue of nephrocystin-4 likewise is located in the proximal region of the flagellum using immunofluorescence microscopy with antibodies against a C-terminal domain of *Chlamydomonas* nephrocystin-4. Furthermore, immunogold electron microscopy of *Chlamydomonas* cells expressing HA-tagged nephrocystin-4 and probed with antibodies against the HA peptide indicated that nephrocystin-4 is specifically localized to the transition zone. Therefore, the location of nephrocystin-4 is conserved among species, suggesting that the function of nephrocystin-4 also is conserved through evolution. We have identified a *Chlamydomonas* insertional mutant in which the nephrocystin-4 gene is completely deleted, and are using it to investigate the function of nephrocystin-4. The mutant flagella look normal, but have defects in both their motile and sensory properties. These defects were specifically rescued by transformation with a wild-type *Chlamydomonas* nephrocystin-4 gene. Reactivated cell models of the nephrocystin-4 mutant cells swim indistinguishably from wild-type cells, suggesting that the motility defect is due to defects in the flagellar membrane rather than the axoneme. Western blotting and immunofluorescence microscopy revealed that the amount of the major flagellar membrane glycoprotein FMG-1B is reduced in the mutant flagella. However, the amounts of other membrane proteins, specifically PKD2 and the mastigoneme protein, are normal. To see if intraflagellar transport (IFT) is altered in the mutant flagella, we carried out western blotting of isolated flagella using antibodies against IFT motors and IFT proteins in both complex A and B. The amounts of IFT proteins in the flagella were not largely affected by the absence of nephrocystin-4. The results suggest that nephrocystin-4 has a role in moving a subset of membrane proteins into the flagellum.

1773/B235

Schmidtea Mediterranea: a Model System for Analysis of Motile Cilia.

P. Rompolas, S. King; MMSB, University of Connecticut Health Center, Farmington, CT

Planarians are free living non-parasitic flatworms that have been studied extensively over the last century for their regenerating properties and more recently as a model for stem cell biology. Planarians are triploblastic, acoelomate and bilaterally symmetrical metazoans with an ectoderm that is largely ciliated. Specifically, the ventral epidermis consists of a single layer of multi-ciliated cells which the organism uses to move over the substrate with a characteristic gliding motion. Motile cilia are also present, but in fewer numbers, on the dorsal surface along with other, more specialized types of ciliary structures that have sensory and secretory roles. *Schmidtea mediterranea* has recently emerged as the planarian species of choice for genetic manipulation due to a sequenced genome, extensive EST libraries and the ability to study gene function using RNAi. Comparative analysis of the *S. mediterranea* genome revealed a great number of highly conserved genes with known ciliary function, including genes present in humans. To test whether this organism might provide a useful model for the analysis of motile cilia, we have used RNAi to down-regulate the expression of a gene that is essential for cilia biogenesis (*IFT88*) as well as genes that have been implicated in the regulation of the outer dynein arm complex such as light chain 1 (*LC1*). Disruption of *IFT88* gene expression results in a dramatic decline in motility at the organismal level due to diminished cilia-based gliding locomotion. Microscopic analysis also revealed a significant decrease in the number of cilia at the ventral surface which is in agreement with the predicted outcome. In our preliminary analysis of the *LC1* knock-down, we observed a considerable decrease in gliding speed, which may result from an altered ciliary beat frequency. Overall, *S. mediterranea* is a highly versatile, easy to maintain and genetically tractable organism that provides an alternative model system for the study of motile eukaryotic cilia and flagella.

Tubulin (1774 – 1783)

1774/B236

Tubulin Binding to Mitochondrial Outer Membrane through Interaction of the C-Terminal "Tails" with VDAC Regulates Respiration- Biophysics, Physiology and Evolution.

J. Sherm[1,2], T. Rostovtseva[1], C. Monge[3], K. Sheldon[1], J. Wolff[2], V. Saks[3], S. Bezrukov[1], D. L. Sackett[1]; [1]NICHD, NIH, Bethesda, MD, [2]NIDDK, NIH, Bethesda, MD, [3]Laboratory of Fundamental and Applied Bioenergetics, Joseph Fourier University, Grenoble, France

The objective of this study is to document the occurrence and biological consequences of tubulin interaction with the mitochondrial outer membrane (MOM) protein VDAC. Mitochondria (mito) have long been known to associate with microtubules (MT) in mammalian cells. Dimeric tubulin has been shown to saturably bind with high affinity to MOM and to co-immunoprecipitate from mito extracts with VDAC, the major channel protein in MOM. We show that tubulin is found in mito preps from a number of cultured human cell lines, and that most of this tubulin runs in native electrophoresis as a high MW complex with VDAC and sometimes Bcl2. Channel conductance of VDAC reconstituted in planar lipid membranes is blocked by addition of nanomolar concentrations of tubulin, but not of tubulin S, which lacks the anionic C-terminal tail peptides of tubulin. Tubulin addition to active mitos increases Km for ADP and lowers oxygen consumption, presumably by restricting ingress of ADP. Thus interaction of tubulin C-terminal tails with VDAC can block nucleotide passage into and out of mito, thereby regulating oxidative phosphorylation. This could occur in all mito-containing cells, and examination of the evolution of tubulin C-terminal sequences revealed high conservation of charge and length of tails throughout eukaryotes. Major exceptions to this in cells with only one alpha or beta tubulin gene are only found in cells that lack mito, in which case tail length and charge may be highly altered. We conclude that tubulin-VDAC interaction has been an important part of cytoplasm-mito regulation and that this has, in part, guided the evolution of tubulin C-terminal tail sequences.

1775/B237

Shrna Stathmin Knockdown in Rat Mammary Tumor Cells Is Associated with an Increase In β-tubulin Class II and MAP4.

S. Lobert[1], C. J. Merkle[3], K. Andersen[1], M. Webb[1], A. N. Vidrine[3], D. W. Montgomery[2]; [1]School of Nursing, University of Mississippi Medical Center, Jackson, MS, [2]College of Medicine, University of Arizona, Tucson, AZ, [3]College of Nursing, University of Arizona, Tucson, AZ

Differential expression of tubulin isotypes or microtubule-interacting proteins (MIPs), such as MAP4 or stathmin may alter the tissue response to antimitotic agents like paclitaxel. We asked whether treatment with paclitaxel when stathmin levels are reduced is associated with changes in β-tubulin isotype or MAP4 mRNA levels. Comparative real-time PCR was used to measure β-tubulin isotype classes II, V, MAP4 and stathmin. Two stable 40-60% stathmin knockdown cell lines (4B6 and 3C4M) were studied. A cell proliferation assay demonstrated that the IC50 paclitaxel concentrations for the wild type and 4B6 cell lines were 80-85 (+/- 15) nM. The 3C4M cell line was about 3-fold resistant to paclitaxel (IC50 259 (+/- 17) nM. There was a significant increase in MAP4 (Student t, $p = 0.02$) and β-tubulin class II (Student t, $p = 0.004$) mRNA in both knockdown cell lines. No change in β-tubulin class V mRNA was observed. The effect of stathmin knockdown on paclitaxel response was measured in a rat model for metastatic mammary tumors (MADB106 cells). The control or 4B6 MADB106 cells were injected into tail veins of Fischer 344 rats and animals were treated with either vehicle or paclitaxel (3 doses over 6 days; 6 mg/kg per IP injection). Fifteen animals were included in each of the 4 groups. Two weeks later, the animals were euthanized and the metastatic lung tumor number and weight determined. No effect of the stathmin knockdown was found for tumor burden, tumor size or tumor number. There was a significant paclitaxel effect on tumor size in both the vector control and 4B6 treatment groups (non-parametric ANOVA, $p < 0.0001$). These in vitro and in vivo data indicate that this model may be useful for examining mechanisms underlying MIP and tubulin isotype interactions in paclitaxel response. (supported by NIH P20-NR07794)

1776/B238

Global Regulation of the Interphase Microtubule System by Abundantly Expressed Op18/Stathmin.

M. E. Sellin, P. Holmfeldt, S. Stenmark, M. Gullberg; Molecular Biology, University of Umeå, Umeå, Sweden

Op18/stathmin (Op18), a conserved microtubule depolymerizing and tubulin heterodimer binding protein, is a major interphase regulator of tubulin monomer-polymer partitioning in diverse cell types in which Op18 is abundant. Here we addressed the question of whether the microtubule regulatory function of Op18 includes regulation of tubulin heterodimer synthesis. We employed two human cell model systems, K562 and Jurkat, combined with strategies for regulatable overexpression or depletion of Op18. Although Op18 depletion caused extensive over-polymerization and increased microtubule content in both cell types, we did not detect any alteration in polymer stability. Interestingly, however, we found that Op18 mediates positive regulation of tubulin heterodimer content in Jurkat cells, which was not observed in K562 cells. By analysis of cells treated with microtubule-poisoning drugs, we found that Jurkat cells regulate tubulin mRNA levels by a posttranscriptional mechanism similarly to normal primary cells, while this mechanism is non-functional in K562 cells. We present evidence that Op18 mediates posttranscriptional regulation of tubulin mRNA in Jurkat cells through the same basic autoregulatory mechanism as microtubule-poisoning drugs. This, combined with potent regulation of tubulin monomer-polymer partitioning, enables Op18 to exert global regulation of the microtubule system.

1777/B239

What Is Tubulin Glutamylation Good for? An Analysis of gGutamylase Function in C. elegans.

N. Peel, K. O'Connell; LBG, NIDDK, NIH, Bethesda, MD

The regulation of microtubule function is crucial to many cellular processes and post-translational tubulin modifications such as tubulin glutamylation are instrumental in regulating microtubule functions. Glutamylation is thought to influence multiple microtubule-dependent processes such as centriole stability, cilia function, vesicle trafficking and neurite outgrowth. The recent demonstration that the tubulin tyrosine ligase like (TTLL) proteins are responsible for tubulin glutamylation opens the opportunity for a comprehensive in vivo analysis of the role of glutamylation. We have shown the presence and subcellular localisation of glutamylated tubulin in C. elegans and we are assessing the function of tubulin glutamylation by analysis of mutant phenotypes after disruption of the C. elegans glutamylases. C. elegans possesses six members of the TTLL protein family including two glutamylation-initiating enzymes, TTLL-4 and TTLL-5. Both TTLL-4 and

TTLL-5 are expressed in the C. elegans embryo and we are assessing their subcellular localizations using GFP fusions and antibodies. We have identified worms mutant for both ttll-4 and ttll-5, which we are using to assess the function of tubulin glutamylation in cell division and development. We find that TTLL-4; TTLL-5 double mutants are homozygous viable and fertile suggesting that tubulin glutamylation is dispensable for cell division. We are currently analyzing microtubule and microtubule associated protein (MAP) behaviour and centriole duplication in the absence of tubulin glutamylation.

1778/B240

Tubulin Tyrosine Ligase as a Possible Vehicle for Fluorescent Labeling of A-Tubulin in Live Cells-Tubulin in Live Cells.

A. Banerjee[1], D. Sackett[2], D. W. McGee[3], S. Bane[1]; [1]Chemistry, SUNY-Binghamton, Binghamton, NY, [2]Laboratory of Integrative and Medical Biophysics, National Institutes of Health, Bethesda, MD, [3]Biological Sciences, Binghamton University, Binghamton, NY

Posttranslational modification of tubulin affects the structure and properties of microtubules. One such modification is the reversible addition of a tyrosine residue to the C-terminus of α-tubulin, which is catalyzed by the enzyme tubulin tyrosine ligase (TTL). Tubulin tyrosination is required for proper cell growth, and disruption of the tyrosination/detyrosination cycle by decreased expression and activity of TTL is associated with increased tumorigenisis and tumor aggression. TTL has high substrate specificity but will accept minor structural variations of tyrosine in vitro. Whether structural variations in a C-terminal tyrosine will affect the function of microtubules in vivo is unclear. We have developed a technique for in vitro labeling of the C-terminus of α-tubulin using TTL in which 3-formyltyrosine (3-fY) is enzymatically incorporated into the protein. Subsequent reaction of the modified protein with hydrazine-containing fluorophores produces a site-specifically labeled tubulin that retains its ability to assemble into microtubules. An added feature of this method is that hydrazone formation shifts the emission maximum to longer wavelength and increases the quantum yield; thus, background fluorescence from unreacted fluorophore is negligible. We wish to extend this method to in vivo labeling of tyrosinated tubulin. CHO cells were incubated with 3-fY, treated with coumarin hydrazine and examined by fluorescence microscopy. Only cells treated with both molecules were fluorescent. Therefore, covalent bond formation between 3-fY and the hydrazine can occur in live cells. 3-Nitrotyrosine is also a substrate for TTL, and the modified tubulin forms normal polymers in vitro. 3-Nitrotyrosine is known to be specifically incorporated into α-tubulin in several types of cells that remain viable. However, under identical growth conditions, 3-fY but not 3-nitrotyrosine was highly toxic to PC-3 cells. The sharp difference in cytotoxicity of the two tyrosine derivatives may be due to different interactions between the C-terminal peptides and non-tubulin proteins.

1779/B241

Exploring the Roles of Neuronal β-tubulin Isotypes.

J. Guo, R. Luduena; Biochemistry, University of Texas Health Science Center at San Antonio, San Antonio, TX

The tubulin molecule is a α/β heterodimer, with both α and β consisting of numerous isotypes encoded by different genes. The differences among the vertebrate β isotypes have been highly conserved in evolution, suggesting that they may have different functional roles. Testing these roles in relatively undifferentiated cells may not be the optimal way of elucidating them. Hence, we have used SK-N-SH neuroblastoma cells, which can be induced to differentiate and grow neurites by treatment with retinoic acid, to examine these roles. These cells express the βI, βII, and βIII isotypes. In general, βI is widespread in normal tissues, βII is the major isotype in nerves and is found in several other tissues, while βIII is found largely in neurons and is relatively rare elsewhere. We have observed that prior to differentiation all three isotypes are present in the cells. After treatment with retinoic acid, the βI, βII and βIII isotypes are present in the neurites and in the cell bodies but βII is particularly concentrated in the neurites. We have created siRNA's specific for each of these isotypes and have found that we can inhibit expression of βI, βII and βIII by 59%, 67% and 72%, respectively. We have found that silencing βI causes about a 50% inhibition in cell proliferation, while silencing βII and βIII cause about a 20% decrease. Cells that have greatly decreased βI are still able to grow neurites of normal appearance. In contrast, cells that have greatly decreased βII have no visible neurites. Cells with greatly decreased βIII exhibit fewer and shorter neurites compared to control cells. From these studies we can propose that βI may play a key role in cell proliferation and that βII is required for neurite outgrowth. Previous work from our laboratory has indicated that βIII may protect cells from oxidative damage. The present work suggests that βIII may also play a role in neurite outgrowth. Supported by grants from the US Army Breast Cancer Research Program (W81XWH-05-1-0238) and the National Cancer Institute (P30 CA54174).

1780/B242

A Single Amino Acid in Class V β-tubulin Is Responsible for Its Effects on Microtubule Disruption and Paclitaxel Resistance.

R. Bhattacharya, F. Cabral; Integrative Biology and Pharmacology, University of Texas Medical School at Houston, Houston, TX

Seven β-tubulin isotypes are expressed in vertebrates. Cellular microtubules are assembled from a subset of these isotypes and their composition therefore varies in a cell and tissue-specific manner. Although the functional significance of producing multiple β-tubulin isotypes is not fully understood, our recent studies have demonstrated that altering the composition of tubulin can affect microtubule assembly, sensitivity to antimitotic drugs, and cell proliferation. An isotype whose expression level has shown potent effects on microtubule assembly is class V β-tubulin (β5). Even though increased expression strongly disrupts microtubule assembly and mitotic progression, low levels of the protein are essential for normal cell division. Overexpression of this isotype also confers resistance to paclitaxel. To identify β5 sequence elements responsible for its phenotypic effects, we compared it to β1, a tubulin isotype that produces no effect when it is overexpressed. By creating chimeras of the two isotypes and using site directed mutagenesis to alter specific residues, we found that two amino acids, S239 and S365, were each able to cause microtubule disruption and confer paclitaxel resistance when introduced into β1. However, a single S239C mutation in β5-tubulin was sufficient to eliminate the distinctive properties of this isotype and caused it to behave like β1 in our assays. The observation that a serine at residue 365 is sufficient to cause microtubule disruption when it is present in β1 but not in β5, indicates that other amino acids that differ between the two isotypes are able to modulate the effects of alterations at this position. Despite the high degree of homology among the β-tubulin isotypes, it thus appears that alterations of at least some amino acids are context sensitive and may cause different phenotypes depending on the isotype in which they occur. The profound effects of substitutions at amino acid 239 in both isotypes leads us to speculate that it may play a critical role in microtubule assembly.

1781/B243

Evidence for the Presence of Gamma-Tubulin in the Nucleolus.

B. Horejsi[1], V. Marková[1], S. Vinopal[1], V. Richterová[1], E. Dráberová[1], V. Sulimenko[1], A. Philimonenko[2], P. Hozák[2], C. D. Katsetos[3], P. Dráber[1]; [1]Biology of Cytoskeleton, IMG ASCR, Prague, Czech Republic, [2]Biology of the Cell Nucleus, IMG ASCR, Prague, Czech Republic, [3]Drexel University College of Medicine and St. Christopher's Hospital for Children, Philadelphia, PA

Gamma-tubulin is a key protein for nucleation of microtubules. It can also participate in regulation of plus-end microtubule dynamics and spindle assembly checkpoint signaling. We have shown previously that in human gliomas and glioblastoma cell lines gamma-tubulin aberrantly accumulated in the cytoplasm (J. Neuropathol Exp. Neurol. 65: 465, 2006). To get further insight into the gamma-tubulin expression in glioblastoma cells, we have performed qRT-PCR experiments that revealed an increase in transcripts for gamma-tubulin 1 as well as gamma-tubulin 2 when compared with normal human astrocytes. Immunofluorescence microscopy of samples prepared under various fixation conditions disclosed that beside microtubule organizing centers, gamma-tubulin was also located in the cytoplasm in the form of large aggregates. Moreover, bright staining for gamma-tubulin was observed in nuclei when acidic fixations were applied. Immunofluorescence with nucleolar markers and marker of Cajal bodies demonstrated accumulation of gamma-tubulin in nucleoli but not in Cajal bodies. Nucleolar staining was inhibited when antibodies were preabsorbed with immunizing peptides. Nucleolar staining of interphase cells was not limited to glioblastoma cells, but was also detected in the other tested cultured cells of various origin. The presence of nucleolar gamma-tubulin was further confirmed by immunoblotting and immunofluorescence of isolated nucleoli. To assess whether exogenous gamma-tubulin is associated with nucleoli, various tagged versions of human gamma-tubulin were prepared. In all tested cases exogenous gamma-tubulin was detected in the nucleoli. Our findings indicate that gamma-tubulin can fulfill, in transformed as well as non-transformed cells, other functions in addition to microtubule nucleation.

1782/B244

γ-Tubulin2 Regulates Mitochondrial Morphology in Neurons.

A. Kubo[1], H. Takagi[1], I. Yao[1], S. Tsukita[2], A. Kubo[3], m. setou[1,4]; [1]Mitsubishi kagaku Institute of Life Sciences, Machida,Tokyo, Japan, [2]Osaka university, Osaka, Japan, [3]Keio University School of medicine, Tokyo, Japan, [4]Hamamatsu University School of Medicine, Hamamatsu, Japan

Γ-Tubulin is a universal component of microtubule organizing centers that is believed to play an important role in microtubule nucleation. Mammalians have two γ-tubulin genes named *Tubg1* and *Tubg2*. *Tubg1* is a ubiquitously expressed γ-tubulin, and *Tubg1* knockout (KO) mice showed early embryonic lethality (Yuba-Kubo *et al*. 2005). On the other hand, *Tubg2* is a neuron specific γ-tubulin. *Tubg2* KO mice were normally developed and fertile, but they showed motor dysfunction depending on nigrostrial pathway (ASCB 2006). To understand these motor defects, we analyzed *Tubg2* KO mice electrophysiologically. γ-Tubulin2 was immunodetected especially in Striatal medium spiny neurons (MSN). When whole cell voltage-clamp recordings were obtained from dopaminergic neurons located within the *Substantia nigra* pars compacta (SNc) in acute slice preparations of mid-brain, mean amplitude of the evoked GABAergic inhibitory postsynaptic currents from the *Tubg2*-KO was significantly smaller than that of WT. The calcium current recorded from cultured *Tubg2*-KO MSN was significantly smaller than that of WT, which was recovered with ATP addition using UV-illuminated Caged ATP. These results indicated that the presynaptic calcium channel dysfunction was induced by ATP insufficiency in GABAergic synaptic input to SNc neurons in *Tubg2* KO mice. To understand the origin of ATP insufficiecy in *Tubg2*-KO MSN, we analyzed mitochondrial morphology and membrane potential of primary cultured MSN. Mitochondrial membrane potential of *Tubg2* KO MSN was significantly depolarized when compared with that of wild type. Surprisingly, very long mitochondria were frequently observed in the *Tubg2*-KO MSN. Co-immunoprecipitation experiment using TUBG2-specific monoclonal antibody showed that γ-tubulin1, GCP2, GCP3, Dynamin1, and DRP1 were bound to γ-tubulin 2 in cytosolic fraction of mouse brain. DRP1 is a well known protein that regulates mitochondrial fission. These results indicate that γ-tubulin2 regulates mitochondrial morphology in neuron together with DRP1. Yuba-Kubo *et al*. Developmental Biology 282: 361-73 (2005), Yuba-Kubo *et al*. 44th ASCB annual meeting Abstract (2006)

1783/B245

An Activated Heterotrimeric G Protein Alpha Subunit Associates with Cytoplasmic Microtubules and Modulates Cellular Morphology.

J. Yu[1], T. Sarma[1], J. Allen[1], M. M. Rasenick[1,2]; [1]Dept. of Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL, [2]Dept. of Psychiatry, University of Illinois at Chicago, Chicago, IL

Alpha subunits of some heterotrimeric G-proteins bind to tubulin and increase dynamic instability of microtubules in vitro. However, only Gαs is translocated in the cytosol after activation. In this study, several biochemical approaches demonstrated that activated-Gαs bound to tubulin and cellular microtubules, and fluorescence microscopy showed that cholera toxin-activated Gαs colocalized with microtubules. The activated-Gαs served as a GTPase activator (GAP) for β-tubulin. As a result, activated-Gαs made microtubules more dynamic, decreasing the pool of insoluble microtubules without changing total tubulin. The amount of acetylated-tubulin (an indicator of microtubule stability) was reduced in the presence of chronically activated Gαs. Mild overexpressing of Gαs in a viral vector showed that activated Gαs could induce neurite outgrowth not only in wild-type PC12 cells, but also PKA-deficient PC12 cells. Wild-type Gαs did not show any affects on the cellular morphology. The results suggest that activated Gαs initiated cellular process formation in both a PKA-dependent and a PKA-independent manner. PKA-deficient PC12 cells were treated with permeable cAMP, the Epac agonist (8-CPT-cAMP) or cholera toxin (which directly activates Gαs). Cholera toxin induced cellular process formation in these cells, suggesting that neurite outgrowth induced by activated Gαs does not require activation of the cAMP pathway. Furthermore, overexpression of a constitutively activated-Gαs in primary cultured hippocampal neuron, demonstrated that activated-Gαs promoted neurite outgrowth and significantly increased the number of spines on the neurites, compared to neurons expressing wild-type Gαs. Thus, it is suggested that activated-Gαs directly regulates microtubule dynamics and promotes neurite outgrowth. These data serve to link G-protein signaling with cytoskeleton dynamics and cell morphology (supported by NIMH, NIDA, DOD)

Dynein I (1784 – 1978)

1784/B246

NDEL1 and LIS1 Interact with the Dynein Motor Domain to Regulate Cortical Microtubule Anchorages.

M. Koonce, I. Tikhonenko; Molecular Medicine, Wadsworth Center, Albany, NY

We previously demonstrated that overexpression of dynein motor domain fragments in Dictyostelium results in a dramatic rearrangement of interphase microtubules (MTs), such that the entire MT array becomes motile in the cytoplasm. This phenotype suggests that dynein participates in forming cortical MT anchorages that normally act to stabilize the radial interphase array. A similar phenotype occurs via disruption of the dynein-binding protein, LIS1 (Rehberg et al 2005, MBC 16:2759-2771). We report here that disruption of the NudE like homolog, NDEL1 in Dictyostelium also produces this motile MT defect. Moreover, we demonstrate that the dynein motor, LIS1, and NDEL1 physically interact with each other in solution. Important here is that these interactions involve domains located in the dynein motor alone, suggesting that LIS1 and/or NDEL1 may directly affect dynein motor activity. Bulk in vitro motility assays that combine motor, LIS1 and NDEL1 polypeptides do not reveal significant differences in MT gliding rates over assays with just the motor alone. One interpretation of this result is that physical association between these three components may be only part of the story, and that posttranslational modifications are also important for motor activity. Overall, our work demonstrates that dynein-LIS1-NDEL function together in a pathway that directly involves the organization of the interphase MT array.

1785/B247

Cytoplasmic Dynein 2 Contributes to the Regulation of Ciliary Length in Tetrahymena Thermophila.

V. Rajagopalan, D. Wilkes, D. Asai; Department of Biology, Harvey Mudd College, Claremont, CA

Cytoplasmic dynein-2 heavy chain is the retrograde motor for intraflagellar transport (IFT) in *Chlamydomonas*, *C. elegans*, and mouse (Pazour et al., 1999; Porter et al., 1999; Wicks et al., 2000; May et al., 2005). Mutations of the dynein-2 heavy chain gene in these organisms result in short, stumpy flagella or cilia. In contrast to the other model organisms studied, the dynein-2 complex in *Tetrahymena thermophila* is not essential for ciliogenesis (Rajagopalan et al., Mol. Biol. Cell, submitted). Knockout cell lines of the *Tetrahymena* dynein-2 heavy chain (KO-DYH2) or the dynein-2 light intermediate chain (KO-D2LIC) continue to produce motile cilia. However, both mutant cell lines contain cilia that are shorter and fewer than those on controls. Interestingly, the lengths of mutant cilia had more variability compared to controls. Short cilia were not unexpected but both mutants produced some cilia that were much longer than normal. In vegetatively growing cultures 7.4 % of KO-DYH2 cilia and 7.8 % of KO-D2LIC cilia were over 7 µm in length while control cells had only 1.1 % of cilia over 7 µm. To determine if the short cilia were due to a ciliary growth rate slower than the rate of cell division, we measured the rate of ciliary growth in non-dividing KO-D2LIC cells. Even after 22 hr of reciliation without cell division the dynein-2 mutants possessed cilia with average lengths significantly less than wild-type, and with significantly more length variation on individual cells. Therefore, the shorter average length of cilia on dynein-2 knockout cells was not simply due to a slower rate of ciliogenesis. We conclude that *Tetrahymena* retrograde IFT is not required for cilia formation but affects the regulation of ciliary length. This work was supported by a grant from the National Science Foundation.

1786/B248

Functional Analysis of Membrane Targeting of Cortical Dynein Receptor Num1 in Budding Yeast.

X. Tang, W. Lee; Biology Department, University of Massachusetts Amherst, Amherst, MA

Pleckstrin homology (PH) domains are small protein modules consisting of 100-120 amino acids commonly found in eukaryotic signaling proteins. A fraction of these proteins are known to localize to plasma membrane, which requires their PH domains. In budding yeast, a PH domain-containing protein Num1 localizes to the cell cortex as punctate patches and is involved in dynein-mediated spindle positioning that drives nuclear migration from mother to daughter cells during mitosis. Deletion of the PH domain causes loss of Num1 patches from the cell cortex and spindle misalignment, resulting in defective nuclear segregation. Although previous studies have shown that the PH domain binds PI(4,5)P2 in vitro with high affinity and specificity, the functional role of this interaction in the dynein pathway has not been tested. Here, we have replaced Num1's PH domain with the last nine residues of Ras2, termed the CaaX motif, which directs protein prenylation and palmitoylation. We found that the CaaX motif fusion fully restored the localization and function of Num1ΔPH mutant. Additionally, Num1ΔPH-CaaX showed partial co-localization with wild-type Num1. These results suggest that the sole function of the PH domain is to target Num1 to the membrane in the dynein pathway.

1787/B249

Distinct Roles of 1α and 1β Heavy Chains of the I1 Inner Arm Dynein of Chlamydomonas Flagella.

S. Toba[1,2], L. A. Fox[3], H. Sakakibara[1], M. E. Porter[4], W. S. Sale[3], K. Oiwa[1,5]; [1]Kobe Advanced ICT Research Center, National Institute of Information and Communications Technology, Kobe, Japan, [2]JSPS Research Fellow, Tokyo, Japan, [3]Department of Cell Biology, Emory University School of Medicine, Atlanta, GA, [4]Department of Genetics, Cell Biology, and Development, University of Minnesota Medical School, Minneapolis, MN, [5]Graduate School of Life Science, University of Hyogo, Hyogo, Japan

I1 inner arm dynein of *Chlamydomonas* flagella, also known as dynein f, is a protein complex composed of two distinct heavy chains (1α and 1β), three intermediate chains and a number of light chains. Studies on flagella of I1 mutants show that I1 dynein plays significant roles in regulation of flagellar motility. Thus, analysis of the enzymatic and motile properties of each motor domain of I1 dynein would be important for understanding flagellar motility. To characterize the roles of individual motor domains, we took advantage of mutant strains lacking either the 1α or 1β motor domains (Myster *et al*., 1999; Perrone *et al*., 2000). SDS-PAGE confirmed that the mutant molecule has all intermediate and light chains found in the wild type except the missing motor domain. Negative-staining electron microscopy showed the one-headed configuration for the isolated 1α and 1β complexes. Steady-state ATPase analysis showed that 1β has 7.6-times higher basal ATPase activity than 1α. Surprisingly, the ATPase of 1α has no activation by microtubules in the range of 0-6 µM tubulin. The microtubule-activated ATPase

of 1β is 14-times higher than the ATPase of 1α. After careful selection of treatment of the glass surface, the 1α and the 1β translocate microtubules smoothly at 1.1 μm/sec and 3.0 μm/sec, respectively, at 1 mM MgATP at 25 °C in conventional *in vitro* motility assays. In summary, the 1β complex has several fold higher enzymatic activity and motility than the 1α complex. The loss of 1β causes more severe impairment in motility of *Chlamydomonas* and failed rescue of microtubule sliding in isolated axonemes by dephosphorylation (Fox *et al*., ASCB abstract issue #1600, 2006). Our *in vitro* data are consistent with these results and suggest that the 1β motor domain makes a greater contribution to microtubule sliding than 1α.

1788/B250

Neuron-Specific 1A Isoform of p150Glued Is Implicated in Spreading and Polarization of Cultured Fibroblast-Like and Epithelial Cells.

O. N. Zhapparova[1,3], S. Bryantseva[1,3], A. Burakov[2], N. Shanina[3], E. Nadezhdina[1,2]; [1]Institute of Protein Research of RAS, Moscow, Russian Federation, [2]Belozersky Institute of Physico-Chemical Biology, Moscow, Russian Federation, [3]Biological Faculty, Moscow State University, Moscow, Russian Federation

Dynactin is a multiprotein complex that enhances dynein activity. One of its subunits - p150Glued - interacts both with dynein and microtubule (MT), ensuring processivity of the whole complex. Along with intracellular transport dynactin is involved in cell migration and neurite growth. A neuron-specific isoform of p150Glued (p135) was described (Tokito, et al., 1996): it has truncated N-terminus and is unable to bind MTs. Here we focus on two other isoforms described by us previously: a full-length p150Glued-1A and p150Glued-1B, that lacks 5-7 exons in MT-binding region. 1B is expressed ubiquitously, though 1A in animals is represented only in neural tissues where it dominates over 1B. In neurons 1A isoform with its enhanced affinity to MTs might ensure long-distance transport. Strikingly 1A isoform is detected also in cultured cells: it is expressed at moderate levels in transformed epithelial HeLa, immortalized epithelial-like Vero, fibroblast-like REF and in primary fibroblasts. This might arise from the peculiarity of cultured cells: their motility and spreading - the processes that implicate MTs and MT-associated proteins, including dynactin. Notably the level of 1A expression is increased in spreading cells (growing in subconfluent compared to confluent monolayer), while the total level of dynactin there is not altered significantly. The highest level of expression is detected in polarized cells at the edge of scratched monolayer. Immunostaining with anti-1A antibodies reveal enrichment of this isoform along MTs directed towards the leading edge. Our preliminary results indicate that protein kinase LOSK/SLK might be one of the potential partners of p150Glued. LOSK was shown to regulate radial organization of microtubules (Burakov et al., 2008) and cell spreading (Wagner et al., 2002). We have disclosed a site of LOSK phosphorylation in p150Glued-1A. p150Glued mutant, mimicking phosphorylation state, restores radial organization of microtubules disrupted by the inhibition of LOSK. Nonphosphorylated mutant and 1B isoform do not affect unfocused MTs. Elucidation of the role of p150Glued-1A and LOSK in cell spreading and migration is a subject of further experiments.

1789/B251

Mat89Bb Coordinates Spermatogenesis in Drosophila by Promoting Proper Localization of Dynein-Dynactin.

M. A. Anderson[1], E. Lee[1], K. Hales[3], T. S. Hays[2], L. A. Lee[1]; [1]Cell & Developmental Biology, Vanderbilt University Medical Center, Nashville, TN, [2]Genetics, University of Minnesota, Minneapolis, MN, [3]Biology, Davidson College, Davidson, NC

Spermatogenesis is a highly dynamic process that utilizes both mitotic and meiotic cell cycles as well as prolonged growth and differentiation programs to generate functional sperm that are capable of delivering their genomic contents to receptive eggs. Due to the complex nature of this process, developing sperm cells must rely on a host of regulatory genes to ensure that they progress from one stage of development to the next. Many of these key players, however, have yet to be identified. Our recent analysis of a *Drosophila* mutant line has revealed that *Mat89Bb*, which encodes a novel, conserved protein, is an essential regulator of spermatogenesis. While mitotic cycles appear to proceed unperturbed in *Mat89Bb* testes, mutant spermatocytes commonly arrest in prophase of meiosis I. Strikingly, arrested cells often contain free centrosomes that have failed to form stable associations with the nucleus. In *Mat89Bb* spermatocytes that evade prophase I arrest, defects in spindle assembly, chromosome segregation, and cytokinesis are frequently observed. Additionally, the normal attachment between the basal body and the nucleus is disrupted in postmeiotic *Mat89Bb* spermatids, resulting in defects in later stages of spermatogenesis. Our data indicate that these defects stem from a loss of dynein-dynactin localization during spermatogenesis in *Mat89Bb* mutants. We have found that dynein-dynactin normally localizes to the nuclear periphery in late G2 primary spermatocytes (prior to the time of centrosome migration from the cortex to the vicinity of the nucleus), to the spindle poles during the meiotic divisions, and to a hemispherical cap adjacent to the nucleus in spermatids. These localizations, however, are seldom observed in *Mat89Bb* testes. Based on our findings, we propose a model in which *Mat89Bb* is required for regulating centrosome/basal body positioning during spermatogenesis via control of dynein-dynactin localization.

1791/B253

Single Dynein Motor Analysis in Living Cell.

M. Schuster[1], U. Theisen[1], S. Rothery[2], N. Severs[2], G. Steinberg[1]; [1]University of Exeter, Exeter, United Kingdom, [2]National Heart & Lung Institute, Imperial College London, London, United Kingdom

Compelling in vitro evidence indicate that numerous cytoplasmic dynein motors are required for the transport of cargo over long distances. However, the minimum number of motors that mediate processive motility in the living cell is not known. In this study we directly observe dynein motility in the phytopathogenic model fungus Ustilago maydis. By GFP-tagging the endogenous copy of the dynein heavy chain and live cell imaging using laser-based wide-field fluorescence microscopy we show here that dynein travels long distances in a bi-directional fashion along single microtubules. Anterograde dynein motility could be blocked by a kinesin-1 rigor allele, suggesting that dynein is a direct cargo kinesin-1 (conventional kinesin). In contrast, retrograde motility is due to intrinsic dynein activity. However, frequency and velocity of both transport directions were almost identical, indicating that the activity of kinesin-1 and dynein is fine balanced. In order to quantify the number of dyneins undergo processive movement, we compared signal intensities of GFP-dynein with the conserved nuclear pore component Nup107 that served an internal calibration standard. In addition, we performed a bleaching step analysis on immobilised dynein in the living cell. Both approaches consistently demonstrated that the majority of the moving dynein signals represent single motors. These results

strongly suggest that in the living cell single dynein motors are capable of taking membranes over large distances. Moreover, as kinesin-1 delivers single dyneins to plus-ends, the activity of both motors is balanced.

1792/B254

Counting the Number of Dynein Molecules at the Plus End and Cortical Num1 Receptor in Budding Yeast.

J. J. Punch, S. M. Markus, W. Lee; University of Massachusetts Amherst, Amherst, MA

Cytoplasmic dynein mediates movement of the mitotic spindle by powering microtubule sliding along the cell cortex in the budding yeast S. cerevisiae. In wild-type cells, dynein is targeted to the microtubule ends for delivery to cortical Num1 receptor sites. In the absence of Num1, dynein accumulates at the microtubule plus end and fails to off-load. Here, we have measured the number of molecules of Dyn1-3mCherry at the plus end and Num1-mCherry at the cortex, using Cse4-3mCherry or Cse4-mCherry (a core kinetochore protein present at 2 molecules per chromosome) as a standard for fluorescence intensity. The number of molecules of dynein at the plus end is 8, while the number of molecules of Num1 at individual cortical patches ranges from 7 to 52. In num1 null cells, dynein at the plus end is increased to16-64 molecules. These measurements indicate that all available binding sites at the plus end are not saturated by dynein in wild-type cells.

1793/B255

Motor and Tail Domains of Dynein Confer Differential Targeting to Microtubule Plus Ends and the Cell Cortex.

S. M. Markus, J. J. Punch, W. Lee; Biology, University of Massachusetts Amherst, Amherst, MA

Cytoplasmic dynein mediates spindle positioning in the budding yeast S. cerevisiae by powering sliding of astral microtubules along the cell cortex. Although previous studies have demonstrated cortical and +TIP localization for the heavy chain of cytoplasmic dynein (Dyn1), the regulation of its recruitment to these sites remains elusive. Here we show that separate domains of Dyn1 confer differential localization to microtubule plus ends and the cell cortex. Using 3GFP and 3YFP tagged constructs expressed at endogenous levels, we found that the N-terminal cargo-binding domain (Tail) of Dyn1 targets dynein to the cortex at Num1 sites, whereas the C-terminal ATPase domain (Motor) of Dyn1 targets dynein to microtubule plus ends. Unlike the full-length protein, the Motor construct is absent from the cell cortex. However, like the full-length protein, the Motor construct requires Bik1/CLIP-170 and Pac1/LIS1 for plus end targeting. Expression of the Motor construct blocks plus end localization of full-length Dyn1, leading to a dominant negative effect on dynein pathway function. Strikingly, genetic assays show that overexpression of Pac1, but not Bik1, rescues the dominant negative effect of the Motor by restoring Dyn1 to microtubule plus ends. Additionally, we observed that the cortical localization of the Tail construct is robust, and present at a significantly higher proportion of cells than full-length Dyn1 (65.1% ± 2.0% versus 17.9% ± 1.9%, respectively), and that this localization is unaffected by deletion of Bik1 or Pac1. Taken together, these results suggest that the Tail cortical association domain is normally masked in the full-length Dyn1 molecule until it is targeted to microtubule plus ends. We propose that Dyn1 may interact with Pac1/LIS1 and other dynein pathway components at the plus end to prime the dynein complex for cortical association and off-loading from microtubule plus ends.

1794/B256

The Microtubule Binding Activity of p150Glued Is Essential for Dynein-Driven Transport.

M. T. Raycroft, M. Werner, L. Casaletti, J. R. Bader, P. Vaughan, K. Vaughan; Biological Sciences, University of Notre Dame, Notre Dame, IN

Dynactin is a multi-subunit complex implicated in cytoplasmic dynein-driven transport of membranous organelles, mitotic chromosomes and other cargos. The p150Glued subunit of dynactin contains a microtubule (MT)-binding domain composed of a CAP-Gly motif and an adjacent basic domain (BD). Despite the widespread conservation of this structure, the requirement for MT-binding remains controversial. To test the hypothesis that MT-binding is important for transport, we engineered mutants that disrupt normal MT-binding and developed a shRNA/rescue assay to replace endogenous p150Glued with each mutant. Assaying ER-Golgi transport first, wild-type GFP-p150Glued was successful in rescuing normal motility. In contrast, a GFP-p150Glued G59S mutant that displays reduced MT-binding incorporated into the dynactin complex but failed to rescue normal ER-Golgi transport. To test if dynamic MT-binding was required, we generated a GFP-p150Glued-MAP4 chimera which exhibited stable MT binding. This construct also failed to rescue ER-Golgi transport but resulted in immobilization of vesicles along MTs. To test the contribution of the BD, a naturally occurring p150Glued isoform with an internal deletion in the BD was prepared. This construct was successful in rescuing ER-Golgi transport but displayed reduced run-lengths for motile vesicles. Because dynactin is also thought to augment dynein-driven chromosome movement during mitosis, we applied the same shRNA/rescue approach to assay kinetochore-MT capture, chromosome movement, and the timing of anaphase onset. Whereas wild-type GFP-p150Glued rescued each aspect of mitotic dynactin function, the G59S mutant disrupted kinetochore-MT capture similar to cells treated with nocodazole. A subset of G59S-rescued cells entered anaphase despite a loss of persistent kinetochore-MT attachments, reflecting a defect in the spindle assembly checkpoint. Together these studies suggest that the MT-binding activity of p150Glued is essential during both interphase and mitosis.

1795/B257

Reconstitution of Complex, ER-Like, Nanotubular Networks, and of Small, Tubulo-Vesicular Transport Entities by Interactions of Cytoplasmic Dynein and Spectrin with Liposomes.

Z. Muresan, V. Muresan; Pharmacology and Physiology, UMDNJ - New Jersey Medical School, Newark, NJ

Cells maintain throughout their lifespan intricate networks of membrane tubes of nanoscale dimensions that participate in the functional compartmentalization of the cell. A typical example is the endoplasmic reticulum (ER). Much smaller tubular entities, derived by extraction from donor compartments (e.g., those emerging from the recycling endosome or the trans-Golgi network) or generated by vesicle fusion (e.g., the ER-to-Golgi transport units), function in intracellular transport. Different mechanisms are thought to underlie the morphogenesis of the complex, tubular ER network, and the formation of the small tubular transport entities that travel along microtubules. Here, we show that the molecular machinery that powers retrograde vesicle motility in neurons can interact with membranes to generate these different types of tubes. We reconstituted in vitro elaborate networks of interconnected membrane tubes (with ER-characteristic ring closures and three-way

junctions), as well as freely moving, stable tubes and tubulo-vesicular clusters, from a mixture of the minus-end motor, cytoplasmic dynein, its regulatory complex, dynactin, spectrin, and liposomes containing acidic phospholipids - in the presence of microtubules and ATP. The tubulo-vesicular clusters contained trains of spherical liposomes attached to a small tube via elastic linkers, likely maintained together through a supravesicular spectrin meshwork that encompasses both the tube and the associated vesicles. Tubes did not form in the absence of spectrin, or in the presence of function-blocking antibodies to dynein. In separate experiments, we show that the recruitment of dynein-dynactin and spectrin from the cytosol to liposomes is stimulated by phospholipase D-induced conversion of neutral phospholipids to acidic forms, and by activation of small GTPases. These results show that similar mechanisms underlie the generation of ER-like tube networks and small tubular transport entities. Both may be generated - and maintained - by the action of soluble microtubule motor complexes and anchoring proteins, which bind to phospholipids, and do not require membrane proteins. Supported by March of Dimes grant 1-FY04-240 and NIH grant R01GM068596.

1796/B258

The N-Terminus of Dynamitin Is a Novel Dynein / Dynactin Inhibitor.

F. Cheong, K. Maier, T. Schroer; Biology, Johns Hopkins University, Baltimore, MD

Dynactin, an activator of dynein and other motors, participates in subcellular activities that range from Golgi positioning to nuclear envelope breakdown and mitotic spindle formation. The dynactin subunit dynamitin (p50) is essential to dynactin's structural integrity. Soluble full-length dynamitin dissociates the subunit p150glued from the Arp1 minifilament in vivo and in vitro, and over-expression of dynamitin causes Golgi dispersion and aberrant mitotic spindles. We have proposed (Maier et al. 2008) that dynamitin mediates disruption of dynactin by binding to endogenous dynamitin and destabilizing dynactin's shoulder structure. We find that the N-terminus fragment of dynamitin N87 (AA 1-87) is a potent inhibitor of dynein/dynactin function, as its over-expession leads to Golgi disruption and aberrant mitotic spindles. Velocity sedimentation analysis of Cos7 cell lysate over-expressing N87 reveals that this fragment can disrupt dynactin, causing release of p150 from the Arp1 minifilament. The ability of N87 to disrupt dynactin in cells is unexpected, since similar recombinant fragments of the dynamitin N-terminus do not disrupt or bind stably to purified bovine dynactin. This suggests that dynamitin AA (1-87) disrupts dynactin through a distinct mechanism from full-length dynamitin. We hypothesize that N87 may bind to and sequester a stabilizing factor of dynactin in cells, leading to a dynactin disruption phenotype. Work aimed at identifying such a factor is currently underway.

1797/B259

Isoform-Specific Phosphorylation Dictates Cargo Binding for Cytoplasmic Dynein in Neurons.

S. Tauhata, B. P. O'Rourke, P. Vaughan, K. Vaughan; Biological Sciences, University of Notre Dame, Notre Dame, IN

Cytoplasmic dynein is a multi-subunit microtubule molecular motor implicated in retrograde axonal transport in neurons from squid to man. It is composed of a pair of catalytic heavy chains (HCs) and a series of intermediate chains (ICs), light intermediate chains (LICs) and light chains (LCs). Several combinations of the dynein ICs and LCs have been suggested as targeting subunits for dynein. A total of six IC isoforms are expressed in neurons through alternative splicing of two IC genes. In a previous work, we performed MS/MS mapping of IC phosphorylation sites and observed isoform-specific phosphorylation in the dynactin-binding domain of the ICs. Because we identified phosphorylation-specific interactions between the IC2-C isoform and binding partners on kinetochores during mitosis (see poster Bader et al., this meeting), we tested the hypothesis that isoform-specific phosphorylation of the ICs dictates cargo-binding in neurons. We optimized blot overlay assays to test the impact of IC phosphorylation on binding to the p150/135Glued subunits of dynactin and other neuronal binding partners. To generate biochemical quantities of phospho-ICs we used squid axoplasm and purified kinases in phosphorylation assays. Binding assays with phospho-ICs demonstrate reduced affinity for p150/135Glued , however the degree of reduction is specific to each IC isoform. In addition, phosphorylation of some isoforms revealed new binding partners. Using computer-based predictions of candidate kinases for IC isoforms, we performed in vitro kinase reactions with individual ICs. Focusing on p38MAPK isoforms, we observed isoform-specific phosphorylation by members of the p38MAPK family. Kinase reactions were more robust for reactions with purified dynein than with recombinant ICs, suggesting that kinase recognition might require intact dynein complexes. Blot overlay assays with the phosphorylated peptides revealed binding to novel binding partners in neuronal extracts. This suggests that isoform-specific phosphorylation of dynein IC isoforms dictates the specificity of binding to cargos in neurons.

1798/B260

Ndel1 Palmitoylation: a Novel Mean to Regulate Cytoplasmic Dynein Activity.

O. Reiner[1], A. Shmueli[1], T. Sapir[1], M. Segal[1], Y. Fukata[2], M. Fukata[2]; [1]Weizmann Institute, Rehovot, Israel, [2]PRESTO, Japan Science and Technology Agency, Chiyoda, Tokyo 102-0075, Japan

Regulated activity of the retrograde molecular motor, cytoplasmic dynein, is crucial for multiple biological activities, and failure to regulate this activity can result in neuronal migration retardation or neuronal degeneration. The activity of dynein is controlled by dynactin, and by the LIS1/Ndel1/Nde1 protein complex, which participates in intracellular transport, mitosis, and neuronal migration. These biological processes are subject to tight multilevel modes of regulation. Palmitoylation is a reversible post-translational lipid modification, which can dynamically regulate protein trafficking. We found that both Ndel1 and Nde1 undergo palmitoylation in vivo and in transfected cells on a conserved cysteine residue by specific palmitoylation enzymes. Ndel1 interaction with cytoplasmic dynein is sensitive to its palmitoylation status. Whereas unpalmitoylated Ndel1 interacts better with dynein, the interaction between Nde1 and cytoplasmic dynein is unaffected by palmitoylation. Furthermore, palmitoylated Ndel1 reduced cytoplasmic dynein activity as judged by Golgi distribution, transport of short microtubule fragments, transport of temperature sensitive VSVG, transport of endogenous Ndel1 and LIS1 from neurite tips to the cell body, and neuronal migration. Our findings indicate for the first time that Ndel1 palmitoylation is a novel mean for fine-tuning the activity of the retrograde motor cytoplasmic dynein.

Conventional Myosin (1799 – 1809)

1799/B261

Regulation of the Localization and Activation of Non-Muscle Myosin II during Cell Division.

J. Beach[2,1], T. T. Egelhoff[1]; [1]Cell Biology, Cleveland Clinic Foundation, Cleveland, OH, [2]Physiology and Biophysics, Case Western Reserve University, Cleveland, OH

Non-muscle myosin II (NM-II) is a hexamer consisting of two heavy chains (MHC), two essential light chains (ELC) and two regulatory light chains (RLC). RLC can be phosphorylated at T18 and S19 and this phosphorylation event has been shown to activate myosin by increasing actin binding, ATPase activity and assembly. Following the metaphase-to-anaphase transition non-muscle myosin II (NM-II) and actin (along with many other proteins) localize to the site of division and create the contractile ring, where NM-II is believed to be responsible for most of the force needed for cytokinesis. However, the mechanism by which NM-II is properly localized and activated remains incompletely understood. Our research aims to identify regulatory mechanisms and/or NM-II binding partners involved in the proper spatio-temporal localization of NM-II by analyzing myosin heavy chain (MHC) truncations fused to GFP in conjunction with confocal microscopy and TIRF microscopy. Our results indicate that neither the myosin head nor light chains are necessary for proper localization and that a region near the C-terminus of the tail that contains the in vitro-identified assembly competent domain (ACD) is both necessary and sufficient for proper localization. Our goal now is to identify whether this construct is localizing via co-assembly with endogenous myosin or via a binding partner.

1800/B262

Nonmuscle Myosin IIA and Myosin IIB with Distinct Roles Regulate Breast Cancer Cell Membrane Extension during Spreading.

V. Betapudi, T. T. Egelhoff; Cell Biology, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, OH

The present work is performed to gain insight into the mechanism of membrane extension, a critical step in the initiation of tumor cell invasion and migration. While actin polymerization generates a mechanistic support or an internal driving force, the formation of focal contacts in the lamellipodium is believed to restrain cell membrane extension. Cells are known to use nonmuscle myosin II-mediated contractile forces in regulating actin and focal contacts dynamics during cell membrane extension via unknown mechanisms. Earlier studies showed enhancement and impairment of cell membrane extension upon depletion of myosin IIA and myosin IIB, respectively. However, myosin IIA and IIB specific roles and the mechanism of cell membrane extension are not clearly understood. The present study reports distinct localization of myosin IIA and IIB with different turnover rates in cell membrane extensions during the spreading of MDA-MB 231 breast cancer cells. Cells expressing myosin IIB and depleted of myosin IIA display a paucity of focal contacts formation whereas, cells expressing myosin IIA, but depleted of myosin IIB displayed focal contacts formation in cell membrane extensions during spreading. This suggests that cells use the myosin IIA-mediated contractile forces to restrain cell membrane extension by stabilizing focal contacts and the myosin IIB-mediated contractile forces to destabilize focal contacts to facilitate cell membrane extension during spreading. Thus, we show that myosin IIA and myosin IIB display distinct mechanistic roles in regulating cell membrane extension during spreading

1801/B263

Myosin II Mediates Local Cortical Tension to Guide Endothelial Cell Branching Morphogenesis and Migration in 3D.

R. S. Fischer[1], M. Gardel[3], X. Ma[2], R. Adelstein[2], C. Waterman-Storer[1]; [1]Laboratory of Cell and Tissue Morphodynamics, NHLBI, Bethesda, MD, [2]Laboratory of Molecular Cardiology, NHLBI, Bethesda, MD, [3]Physics, University of Chicago, Chicago, IL

Angiogenesis is a critical process in both development and disease, where it represents a prime target for therapeutic intervention. A key feature of angiogenesis is directional control of endothelial cell (EC) morphogenesis and movement. During angiogenic sprouting, endothelial "tip cells" directionally branch and migrate from existing vessels in response to biochemical cues such as VEGF or hypoxia. Tip cell branching is mediated by directional protrusion of subcellular pseudopodial branches. We sought to understand how EC pseudopodial branching is locally regulated to directionally guide angiogenesis. To achieve this, we first established a model system that produced physiologically relevant cell behavior. We found that in in vitro 3D collagen gels, primary mouse ECs (both as single cells and at tips of nascent tubes) mimic the EC morphodynamics of angiogenic tip cells in living zebrafish. We demonstrate that increased ECM stiffness and ROCK-mediated myosin II activity inhibit EC pseudopodial branch initiation and favor enhanced branch retraction, concomitant with an increase in directional persistence of EC migration. Live cell imaging of ECs in 3D collagen matrices demonstrate that GFP-myosin IIB is localized at the EC cortex, where it found in both dynamic web-like arrays and in linear F-actin bundles reminiscent of stress fibers seen in stiff 2D cultures, despite the low stiffness of the collagen matrix (>100 Pa). FRAP demonstrates that a greater percentage of GFP-myosin IIB is dynamic in soft substrates which promote branching as compared with stiffer substrates which inhibit branch initiation. Local depletion of cortical myosin IIB precedes branch initiation events, and initiation of branches can be induced by local inhibition of myosin II activity. Thus, we propose that stochastic, local downregulation of myosin II-mediated cortical tension allows pseudopod initiation, which thereby regulates EC branching and hence guides directional migration during angiogenesis.

1802/B264

Synergistic Effect of Ablation of Nonmuscle Myosin II-B and II-C on Karyokinesis in Mouse Cardiac Myocytes.

X. Ma, S. Kawamoto, M. Conti, S. Jana, R. Adelstein; LMC, NHLBI/NIH, Bethesda, MD

Three isoforms of NM II, NM II-A, II-B and II-C are expressed in mammals. Ablation of NM II-A in mice leads to embryonic lethality by E6.5 with disorganized germ layer formation. Mice ablated for NM II-B (B^-/B^-) die by E14.5 with defects in heart and brain development. The cardiac defects include a decrease in the number of cardiac myocytes which can be attributed in part to abnormalities in cytokinesis. To understand the function of NM II-C in vivo, we generated mice ablated for NM II-C. The C^-/C^- mice are viable and show no obvious abnormalities during development, but show mild phenotypic changes in the heart, including a decrease in cardiac function as measured by MRI at 6 months and moderate myocyte hypertrophy at one year. The presence of mild cardiac abnormalities in C^-/C^- mice prompted us to cross these mice with II-B hypomorphic mice ($B^{\Delta I}/B^{\Delta I}$) which express only 12% of the wild type levels of NM II-B in the heart. The homozygous offspring of this mating ($B^{\Delta I}C^-/B^{\Delta I}C^-$) develop cardiac myocyte hypertrophy and interstitial fibrosis at 6 months, a time when both C^-/C^- and $B^{\Delta I}/B^{\Delta I}$ mice show no such abnormalities. To further examine this effect in cardiac myocytes, we generated II-B and II-C

double knockout mice (B^-C^-/B^-C^-). Similar to B^-/B^- mice, B^-C^-/B^-C^- mice died by E14.5 with a hypoplastic heart. In addition to a failure in cytokinesis, B^-C^-/B^-C^- cardiac myocytes also showed major defects in karyokinesis. During mitosis many B^-C^-/B^-C^- myocytes demonstrate defects in spindle formation and chromatid separation. While defective karyokinesis in the cardiac myocytes was occasionally observed (~15%) in B^-/B^- mice, the frequency was increased to 90% in B^-C^-/B^-C^- mice. The requirement for NM II in karyokinesis was further demonstrated in cultured HL-1 atrial myocytes (courtesy of William Claycomb, LSU) using siRNA knockdown of NM II or blebbistatin. Unlike the cardiac myocytes where NM II-B is the predominant isoform and II-A is not detected, HL-1 cells express 95% NM II-A and 5% II-B. An additive effect on karyokinesis in HL-1 cells was seen following siRNA knockdown of NM II-A and II-B. In summary we present evidence that NM II isoforms play a role in cardiac myocyte karyokinesis in addition to cytokinesis.

1803/B265

The Nonmuscle Myosin II-A Motor Domain Is Important for Early Mouse Embryonic Development.

A. Wang, X. Ma, R. Adelstein; LMC, NHLBI, Bethesda, MD

In vertebrates three genes, Myh9, Myh10 and Myh14 encode three different isoforms of the nonmuscle myosin II heavy chain (NMHC II). The motor activity of nonmuscle myosin II (NM II) resides in the N-terminal globular head domain and filament formation resides in the C-terminal rod domain. Previous work has shown that ablation of NM II-A in mice results in lethality by E6.5 with defects in cell-cell adhesion and a failure to produce a competent visceral endoderm. To understand the function of NM II-A during development we used homologous recombination to generate 4 different mouse lines:1-we replaced NM II-A with NM II-B by "knocking in" cDNA encoding human NMHC II-B into the II-A locus, thereby ablating II-A and placing NMHC II-B under control of the endogenous II-A promoter (Ab*/Ab* mice). 2-we replaced endogenous NM II-A by knocking in two chimeric NMHCs, one encoding the N-terminal motor domain of NMHC II-A fused to the C-terminal II-B rod domain (Aab/Aab mice), 3-and another encoding the N-terminal domain of NMHC II-B fused to the C-terminal domain of II-A (Aba/Aba mice). 4-For control mice we inserted cDNA encoding NMHC II-A into the II-A locus. Replacing NM II-A with II-B (Ab*/Ab*) allows normal development of the visceral endoderm, gastrulation, organogenesis and survival to E9-10. These mice show a delay in embryonic turning with defects in the vasculature and endocardium. Aba/Aba mice die at the same age as Ab*/Ab* mice indicating that the N-terminal motor domains are interchangeable between II-A and II-B up to E9-10 despite differences in the kinetic properties of the motors. Aab/Aab mice survive beyond E12 with a normal vasculature but with a hypoplastic heart and an aorta overriding a ventricular septal defect. Thus, the N-terminal II-A motor domain has extended the developmental period from E9-10 to E13, displaying its importance for the early embryonic development.

1804/B266

Kinetic Characterization of the Myosin IIA with an N-Terminal GFP Fused Regulatory Light Chain.

A. Kengyel, J. Sellers; Laboratory of Molecular Physiology, National Heart Lung and Blood Institute, Bethesda, MD

Nonmuscle myosin II plays a crucial role in a variety of cellular processes (e.g. polarity formation, cell motility, cytokinesis). It is composed of two heavy chains, two regulatory light chains (RLC) and two essential light chains (ELC). The ATPase activity of the myosin II motor domain is regulated through phosphorylation of the RLC by the myosin light chain kinase (MLCK). To study the myosin function in cellular processes, GFP fused regulatory light chain (GFP-RLC) is widely used, however the exact kinetic properties of the GFP-RLC myosins are poorly described. In our present work we coexpressed N-terminal GFP fused RLC with an HMM-like fragment of nonmuscle myosin IIA and ELC to characterize its phosphorylation dynamics and in vitro kinetic properties. Our preliminary data show, that the GFP-RLC became fully phosphorylated, but at half the rate as the wild type RLC. The steady-state ATPase activity of the GFP-RLC myosin significantly decreased: Vmax ~ 0.2 s-1 comparing with ~ 0.4 s-1 for wild type, although the velocity in actin gliding assay show a similar pattern to the wild type. The elongated phosphorylation time does not explain the difference in the ATPase activity, so other inhibitory mechanisms are also possible. However a slower phosphorylation rate could be significant in cells, where the degree of phosphorylation of nonmuscle myosin is determined by an equilibrium between various kinases and a phosphatase.

1805/B267

Single Molecule Kinetic Measurements of Non-Muscle Myosin IIB Using Optical Tweezers.

A. Nagy[1], Y. Takagi[1], M. Kovacs[2], E. Homsher[1], J. R. Sellers[1]; [1]LMP, NIH/NHLBI, Bethesda, MD, [2]Department of Biochemistry, Eotvos University, Budapest, Hungary

Non-muscle myosin IIB (NMIIB) is a cytoplasmic myosin which is ubiquitously expressed in eukaryotic cells, particularly in the central nervous system and cardiac tissues. Our previous solution kinetic study of the single headed, subfragment-1 (S1) construct of NMIIB showed that this myosin spends a significant amount of its actomyosin ATPase cycle in the strongly bound state. In the presence of actin, the rate of ADP release is slow (~0.35 s-1), comparable to the steady-state ATPase rate (0.13 ± 0.01 s-1). Furthermore, ADP-affinity for NMIIB is the highest reported so far for the myosin super-family (<0.15 μM). These unique kinetic parameters are advantageous for NMIIB, whose function in vivo has been shown to be associated to cortical tension generation and maintenance. To study the kinetics of single molecules of NMIIB-S1 constructs interacting with a single actin filament, we used a dual-beam optical tweezer apparatus to perform single molecule kinetic/mechanical studies using the 'three-bead' assay. We measured the lifetimes of unitary actomyosin interactions and determined the actin-detachment kinetics with varying ATP concentrations. Optical trapping results showed that at physiological ATP concentration (1 mM), the rate of detachment of acto-NMIIB-S1 interactions was ~0.3 s-1, similar to the ADP release rate and steady-state ATPase rate reported from solution kinetic studies. Decreasing the ATP concentration (10 μM) did not alter this rate of detachment. Additional single molecule experiments were performed by adding excess ADP into the NMIIB environment (1 mM ADP, 1 mM ATP), which prolonged the strongly bound states further, exhibiting behavior of a molecule with high ADP affinity. We will discuss these kinetic measurements, as well as the mechanical measurements, extracted from the optical tweezers experiments in the context of NMIIB's role as a tension generating motor in vivo.

1806/B268

Critical Residues for Assembly of Drosophila Non-Muscle Myosin II.

D. Ricketson, R. Frei, N. Fewkes, K. Prehoda; University of Oregon, Eugene, OR

Non-muscle myosin II generates contractile forces for processes such as cytokinesis, motility, and polarity. Contractility requires assembly into bipolar mini-filaments through electrostatic interactions between coiled-coil tail domains of the heavy chains. Analyses of myosin II from various organisms have revealed "assembly domains" within the C-terminal portion of the tail domain that mediate filament formation. However, it has been unclear precisely how assembly domains interact with one another to form the mini-filament structure. To understand assembly domain interactions, we identified residues critical for filament assembly within the Drosophila non-muscle myosin II assembly domain by analyzing point mutants with in vitro pelleting and S2 cell cleavage furrow targeting assays. We found that single point mutations in specific positively charged regions completely disrupt filament assembly. Surprisingly, none of the negatively charged regions within the assembly domain are required for assembly. Most of the mutations in positively charged residues that disrupted filament assembly clustered within a 15-residue segment (1880-1894) that appears to form a critical interaction surface. Using this information, along with known geometrical constraints and electrostatic calculations, we constructed a structural model of the bipolar mini-filament. This model features one favored anti-parallel tail overlap and multiple slightly less stable alternative overlaps. The ability of the positive segment to interact with multiple negative regions explains the lack of required negatively charged residues in the assembly domain. To our knowledge, this structural model of the 8.58 MDa non-muscle myosin II bipolar filament is consistent with all physical observations and provides a framework for understanding the detailed mechanism by which this fundamental cellular structure is generated.

1807/B269

Apical Constriction of Squamous Amnioserosa Cells in Drosophila: Characterizing Contractile Myosin Meshworks and Possible Regulation by PAR6/atypical Protein Kinase C.

D. J. David, T. Harris; Cell & Systems Biology, University of Toronto, Toronto, ON, Canada

Apical constriction is required for different types of cellular invagination during Drosophila embryogenesis. Myosin regulation is crucial for this constriction and is well characterized in columnar cells but the nature of myosin dynamics in squamous cells is less clear. We sought to characterize myosin contraction in squamous amnioserosa (AS) cells during dorsal closure (DC). During DC, large, flat AS cells apically constrict and are amenable to live-imaging with spinning disk microscopy. We find that regulatory myosin light chain (spaghetti-squash, sqh)-GFP begins to form contractile meshworks in AS cells at the onset of DC. Throughout DC, the meshworks contract with regular periodicity. In the flat AS cells of early DC, sqh-GFP meshworks are present and contract over both apical and basal cortexes. In the more cuboidal AS cells of mid-DC, sqh-GFP dynamic meshworks are predominantly apical while basal sqh-GFP is organized in a structurally distinct and static meshwork. This suggests regulation to distinguish apical myosin contraction from basal. To examine possible regulators of the dynamic apical myosin, we looked at apical regulators PAR6 and aPKC. During DC, apical build-up of PAR6/aPKC in AS cells is concomitant with that of myosin. PAR6-GFP is present cortically and in persistent apical surface puncta. PAR6 apical puncta coalesce with AS contraction and are absent basally, suggesting a possible link between apical myosin meshworks and PAR6 localization. To investigate a functional link between myosin and PAR6/aPKC, loss-of-function studies were done. Loss of par-6 or apkc enhances dorsal hole size in cuticle phenotypes of myosin mutants, suggesting a genetic link between myosin and PAR6/aPKC during DC. Our observations of squamous AS cells constricting via dynamic myosin meshworks localized with PAR6/aPKC are similar to observations made by others in C. elegans embryos showing myosin and PAR proteins localized to apical surface meshworks in cuboidal cells. This suggests a general mechanism of apical constriction in non-columnar cells via apical myosin meshworks. These meshworks must be regulated to specify their apical activities and this may involve the apical regulators PAR6/aPKC.

1808/B270

Myosin Heavy Chain Kinase C Mediates Dictyostelium Cell Adhesion via a Myosin II-Independent Mechanism.

A. Chowdhury, A. Franklin, J. Underwood, L. Hyatt, P. Steimle; Department of Biology, University of North Carolina at Greensboro, Greensboro, NC

Studies in Dictyostelium have demonstrated that myosin II plays a role in cell adhesion by driving localized detachment of the cell from substrate. In Dictyostelium, myosin II filament disassembly is driven by phosphorylation of the myosin II heavy chain (MHC) "tail" via the activities of three structurally-related MHC kinases: MHCK-A, -B, and -C. All three MHCKs share homologous catalytic and WD-repeat domains. In the studies presented here we examined the roles that the individual MHCKs play in regulating cell adhesion. Assays in which cells were subjected to increasing rotational speeds (up to 200 rpm) revealed that cells lacking MHCK-C exhibit significantly reduced adhesion to substrate when compared with cells lacking either MHCK-A or -B. By contrast, we found that cells over-expressing MHCK-C exhibit strikingly stable adhesion to substrate, with nearly 90% of these cells remaining attached at 200 rpm. At that speed far fewer cells lacking MHCK-A (55%) or - B (15%) remained attached. Surprisingly we found that MHCK-C mediated adhesion is myosin II independent since the presence of consititutively assembled myosin II does not rescue the hyper-adhesive state induced by overexpression of MHCK-C. We further show biochemically that a truncation of MHCK-C lacking the N-terminal WD-repeat domain (MHCK-C-Δ-WD) is unable to phosphorylate MHC in vitro. Cells over-expressing MHCK-C-Δ-WD display elevated adhesion levels comparable to those observed for cells over-expressing full-length MHCK-C. Thus, even when MHCK-C is expressed as a truncation (MHCK-C-Δ-WD) that cannot control myosin II activity, it can still drive cells to form highly stable attachments to substrate. Taken together, these studies support a model in which MHCK-C, which co-localizes with myosin II at the cell rear, can facilitate cell adhesion by catalyzing the disassembly of myosin II bipolar filaments and by driving the formation of myosin II-independent attachments to substrate. In the context of cell migration, these activities of MHCK-C may play a role controlling the rate and direction of cell locomotion during chemotaxis or multicellular development.

1809/B271

Mass-Spectroscopic Analysis of Autophosphorylation Sites in Dictyostelium Discoideum Myosin Heavy Chain Kinase A.

V. Rai[1], T. T. Egelhoff[1], P. Steimle[2]; [1]Cell Biology, Cleveland Clinic foundation, Cleveland, OH, [2]Department of Biology, University of North Carolina at Greensboro, Greensboro, NC

Dictyostelium discoideum myosin II heavy chain kinase A (MHCK A) plays key role in regulation of myosin II assembly and localization through phosphorylation of three residues located near the C-terminus of the myosin heavy chain. Phosphorylation of the threonine residues drives myosin II from a filamentous to a monomeric state and plays a central role in inhibiting the cellular activity of myosin II. MHCK A has three distinct domains: an 50-kDa N-terminal coiled-coil region, 35-kDa central catalytic domain, and a C-terminal WD-repeat domain. Earlier studies have shown autophosphorylation as a critical activator for MHCK A in vitro. To understand the biochemical mechanism by which autophosphorylation activates MHCK A, and the in vivo roles of this autophosphorylation, we have initiated mass spectroscopy studies to map the autophosphorylation sites. Residues were identified in the catalytic domain and just upstream of catalytic domain, which displayed increased phosphorylation with increasing time of autophosphorylation period. These sites were subjected to site-directed mutagenesis to convert these residues to non-phosphorytable alanine residues or aspartic acid residues to mimic phosphorylation. Biochemical analysis and in vivo studies are in progress to assess the behavior of these mutant kinase constructs.

Cell Motility I (1810 – 1831)

1810/B272

One-Dimensional Topography Underlies Rapid Three-Dimensional Cell Migration.

A. D. Doyle, F. Wang, K. Matsumoto, K. Yamada; LCDB, NIH/NIDCR, Bethesda, MD

Current concepts of cell migration were established in regular two-dimensional (2D) cell culture, but the roles of topography are poorly understood for cells migrating in 3D fibrillar extracellular matrix. We use a novel micropatterning technique termed micro photopatterning (μPP) to identify functions for 1D fibrillar patterns in 3D cell migration. In striking contrast to 2D, cell migration in both 1D and 3D is rapid, uni-axial, independent of ECM ligand density, dependent on contractility, and less dependent on Rac. 1D and 3D migration are also characterized by an anterior microtubule bundle with a posterior centrosome. We propose that cells migrate rapidly through 3D fibrillar matrices by mechanisms characteristic of 1D rather than 2D matrices.

1811/B273

Architectural Algorithmic Abstraction to Understand 4-Dimensional Matrix-Driven Cell Motility.

M. C. Tamby[1], E. S. Savig[1,2], A. Viesca[1], J. E. Sabin[1,2], P. L. Jones[1,2,3]; [1]Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA, [2]Non-Linear Systems Organization and Department of Architecture, University of Pennsylvania, Philadelphia, PA, [3]Pathology & Laboratory Medicine, University of Pennsylvania, Philadelphia, PA

Cell motility represents an abstract endpoint of the convergence between numerous intrinsic and extrinsic effectors, including cell adhesion to the extracellular matrix (ECM). The means to quantify and visualize how the ECM controls cell motility, however, remain highly subjective, especially within 4-D systems. To redress this, we have developed novel computational tools using approaches used in architectural digital design. As a model biological system, normal human pulmonary artery (PA) smooth muscle cells (SMCs) were cultivated either on 3-D fibrillar or non-fibrillar type I collagen thin films that mimic the micro-environment of a normal or hypertensive PA respectively. Using real-time image acquisition, dynamic PA SMC edge morphologies were manually traced and passed through a series of scripted algorithms that yield an unbiased "signature package" of visual and numerical information describing cell motility. In developing the digital tools, our process of continuously abstracting 3-D geometric representations of cellular edges gave rise to an array of systematic methodologies that inherently provide equivalent numerical data used to quantitatively describe the differences in cell motility observed on native and denatured collagen. Metrics derived through this process include the furthest reach of a cell from its center of mass, velocity of cellular edge points, edge fragmentation and cell-cell edge proximities. This computational approach provide ways of describing and representing morphological change in a hierarchical manner such as filopodial wavering, fibrillar alignment, cell repulsion and attraction as well as and central cohesion, at the scale of the local cell edge, the entire cell, and cell-to-cell interactions. The discovery of such behaviors was prompted during the interpretation of visual abstractions, as they reveal relationships between the cell and its micro-environment.

1812/B274

Influence of Tissue Structure on Cell Movement Patterns in Halocyinthia Roretzi.

Y. Kato; Faculty of Engineering, Tohoku Gakuin University, Tagajo, Japan

Objective: Tissue structures exhibit various patterns: smooth, rough, flat, raised, and sinking. The pattern and ability of cell movement is directly related to the characteristics of tissue structure. Certain cells in Halocynthia roretzi have been known to move around in the tissue. Moreover, the tissues of H. roretzi exhibit varied structural characteristics. In this study, I evaluated the influence of tissue structure on cell movement pattern, using the muscle and tunic of H. roretzi. Materials and Methods: The tunic and muscle were observed using optical and electron microscopy. The cells were collected from each tissue by centrifugation and doped with quantum dots. These cells were observed using confocal microscopy and then placed on a glass bottom dish containing artificial sea water at room temperature. Results and Discussion: The muscle was covered with a flat fiber sheet, and did not exhibit any obvious rise or sinking. The cells on the muscle fiber and the sheet were clearly observed. Microscopic observations revealed that the cell changed its shape quite easily, but the area of the cell in the sample slice used for observation remained unchanged. On the contrary, the tunic exhibited an arrangement of fibers in several patterns: comb-like, loose, and random arrangement. The area of the cell in the sample slice changed drastically. Moreover, four-dimensional spatio-temporal scanning showed that the quantum dot in the cell exhibited three-dimensional pattern of movement. The behavior of the cell in the tunic differed from its behavior in the muscle under the same geometrical situation. Thus, the cell behavior is decided by its inhabitancy rather than by its ability to sense the circumstances Conclusion: The characteristics of cell movement are decided by the geometrical pattern of cell inhabitancy.

1813/B275

Solid Boundaries Decrease the Propulsive Force of Swimming Spermatozoa.

E. Lauga[2], L. Shi[1], J. M. Nascimento[3], T. Chen[1], M. Berns[4,1,3]; [1]Bioengineering, University of California, San Diego, La Jolla, CA, [2]Department of Mechanical and Aerospace Engineering, UCSD, La Jolla, CA, [3]e Department of Electrical & Computer Engineering, UCSD, La Jolla, CA, [4]Beckman Laser Institute, UCI, Irvine, CA

Using a 1064nm wavelength optical tweezer operating up to 420 mw focused by a Zeiss 40X NA 1.3 microscope objective, we measure the effect of a nearby flat solid boundary (glass coverslip, thickness 0.15 mm) on the propulsion of human spermatozoa. The optical trap was focused at 0, 20, 40, 60, 100 microns above the solid surface, and the escape force measured at each depth. Based on over 900 individual spermatozoa studied, we demonstrate that their propulsive force, defined as the minimum force required to hold the cell in place by the optical trap, increases with the distance between the cell body and the nearby surface. These results, when considered in the light of previous theoretical work on the hydrodynamics of swimming cells near surfaces, indicate that human spermatozoa modify their flagellar beat patterns near surfaces.

1814/B276

Comparative Analysis of Light-Mediated Motility Responses in Diatoms.

S. Cohn, J. E. Sbarboro, A. C. Pike, Z. B. Kaplan; Biology, DePaul University, Chicago, IL

High irradiance light at the leading tips of moving diatoms induces a reversal of cell direction (Diat. Res. **14**:193; **19**:167) with species displaying characteristic sensitivities to wavelength and irradiance levels. Such behavior helps cells control their position in aquatic environments, maximizing their light gathering ability while minimizing their exposure to harsh conditions or predators. Our lab has been characterizing the light response in three diatom species: *Craticula cuspidata*, *Stauroneis phoenicenteron*, and *Pinnularia viridis*. Diatoms from these species were irradiated at their leading or trailing ends at a variety of irradiance levels and three different wavelengths (blue-470nm; green-550nm; red-650nm). *Craticula* and *Stauroneis* were most sensitive to blue light irradiations at their leading ends, causing rapid direction changes (changing direction in 13±2 and 45±8 sec, respectively, compared to unirradiated controls of 51±4 and 113±17 sec). Irradiations at the leading or trailing ends also caused temporary repression of subsequent direction changes. For example, fifteen seconds after being irradiated at the leading end, cells showed a significant increase in the time to respond to subsequent leading end exposures. The response time for such secondary green irradiations of *Craticula* cells increased from 16 sec to 52±13 sec; with blue irradiations the second response time increased from 10±1 to 22±3 sec. Similarly, trailing end irradiations also repressed the ability of cells to change direction. Both leading and trailing end repressions lasted about 60 sec. We also investigated the wavelengths and irradiance levels required to induce cell movement into areas of light (typical of low light levels) or out of the light (typical with high light irradiations). In blue light, *Craticula* cells showed decreasing out-of-light responses as irradiances dropped from 10^5 to 10^3 μmol/m^{2}sec, with a peak of into-light response at around 10^4 μmol/m^{2}sec. Red irradiations in *Stauroneis* induced into-the light responses down to 10^3 μmol/m^{2}sec. This work was supported by DePaul University Research Council, the DePaul College of Liberal Arts & Sciences, and equipment purchased through NSF Grant IBN-9982897.

1815/B277

Cytoskeletal Regulation of Traction Forces at Adhesive Contacts.

T. Nguyen, S. Yamada; Biomedical Engineering, University of California, Davis, CA

The regulation of cell adhesion is essential for tissue development and homeostasis. Stable cell junctions maintain normal epithelial tissue architecture, but during embryogenesis, tissue regeneration, and cancer cell migration, adhesive contacts loosen and become traction sites for cellular rearrangement. Cellular traction forces are generated by the actin cytoskeleton, and the remodeling of the actin network is thought to regulate the strength and dynamics of cell adhesion. However, the precise molecular linkage between the adhesion complex and the actin cytoskeleton remains elusive. The objective of our study is to define the mechanisms by which adhesion complexes and the actin cytoskeleton regulate cellular traction force. This requires simultaneous analysis of mechanical force and protein interactions at adhesive contacts. To quantitatively analyze traction forces, we fabricated an elastic micro-pillar substrate that is compatible with high-resolution confocal microscopy. We coated the pillar tips with collagen, fibronectin, the extracellular domains of E-cadherin, or N-cadherin to mimic sites of cell-matrix or cell-cell contact. To analyze protein interactions at force bearing adhesive contacts, various proteins in adhesion complexes and actin were GFP-tagged and stably transfected into normal epithelial cells. These cells were seeded onto elastic pillar substrates and examined with a spinning disk confocal microscopy system with Fluorescence Recovery After Photobleaching module. With collagen or fibronectin-coated pillars, cells exerted greater traction force compared to that of cadherin-coated pillars, indicating that these adhesive receptors may signal distinct modes of force transduction at cell-matrix or cell-cell contacts. Often, GFP-actin organized to form a circumferential ring around pillar tips, but on deforming pillars, GFP-actin was asymmetrically distributed. Surprisingly, after photobleaching actin bundles near force-bearing adhesive contacts, we observed rapid fluorescence recovery of GFP-actin. Our result suggests that maintenance of cellular traction force or tension is mediated by dynamic protein linkage and does not require stable actin networks as previously suspected.

1816/B278

Differential Effects of Protease Inhibitors in 2-Dimensional and 3-Dimensional Cell Invasion Assays.

K. I. Hulkower[1], S. Soltaninassab[1], K. M. Anhalt[1], R. L. Herber[1], K. Moin[2], B. F. Sloane[2]; [1]Platypus Technologies LLC, Madison, WI, [2]Department of Pharmacology & Karmanos Cancer Institute, Wayne State University School of Medicine, Detroit, MI

Tumor cell invasion through an extracellular matrix has long been established as a key component in the metastasis of cancer. Previous studies have shown that inhibitors of a broad range of proteases, such as matrix metalloproteinases (MMPs), urokinase and cathepsins B and L, are effective in reducing the invasion of tumor cell lines in 2-D assays that employ matrix coated porous transmembrane filters. We have recently developed a 3-D invasion assay in a 96-well plate format wherein cells were seeded onto a layer of basement membrane extract (BME) in an annular fashion around a centrally placed silicone stopper within the plate well. Adherent cells were covered with a second layer of BME once the stoppers were removed. Cells were then permitted to invade three dimensionally into the analytic zone formed upon the

removal of the stopper. We confirmed the presence of proteases in HT-1080 cells and in HT-1080DM cells, a cell line that was selected for growth in serum free, defined medium and studied them in this 3-D invasion assay. The presence of proteases was detected via immunostaining and by a whole cell enzymatic activity assay. While we demonstrated that the enzymatic activity of the HT-1080 and HT-1080DM cells could be abolished by treatment of the cells with E-64 and leupeptin in whole cell assays of cathepsin B, we found that these inhibitors, as well as inhibitors against urokinase and MMPs, were ineffective in blocking invasion and invadopodia formation by these cells in our 3-D invasion assay. However, inhibitors of actin-myosin components of the cytoskeleton were effective in blocking invasion in our assay. We conclude that cells can utilize different mechanisms to maneuver and invade through BME when subjected to 2-D versus 3-D conditions. Our findings that cell invasion in a 3-D assay can occur independently of protease activity may be more physiologically relevant for drug discovery efforts as these results substantiate the lack of efficacy of protease inhibitors observed in in vivo settings.

1817/B279

Transmission of Cytoskeletal Forces to the Substrate during Cell Migration.

M. F. Fournier, R. Sauser, A. B. Verkhovsky; Laboratory of Cell Biophysics, EPFL, Lausanne, Switzerland

During cell migration, forces generated by actin cytoskeleton are transmitted through adhesion complexes to the substrate. To investigate the mechanism of force generation and transmission, here we analyze simultaneously actin movement and substrate deformation in a simple model system of persistently migrating fish epidermal keratocytes. To quantify the force transmission from actin cytoskeleton to the substrate we introduced a new parameter: actin/substrate viscous friction, defined as the ratio of the substrate stress to the relative velocity of actin with respect to the substrate. Friction maps showed strong connection between cytoskeleton and the substrate under the leading lamellipodia and sides of the cell, and weak or no connection under the cell body. Friction patterns correlated with the changes in cell motion, e.g. turns. To evaluate the specific contributions of actin assembly and actomyosin contraction to force generation and transmission we used cytochalasin D and blebbistatin, which inhibit actin polymerization and the activity of myosin II, respectively. Cytochalasin D treatment resulted in the arrest of the leading edge, but the cell body continued to translocate for a few seconds, and significant forces were registered at the substrate during this period. These forces could be considered as due to actomyosin contraction only. On the other hand, blebbistatin-treated cells migrated slower and exhibited no measurable forces and friction at the substrate. After addition of cytochalasin D, blebbistatin-treated cells slowed down simultaneously at the front and the cell body, suggesting that in this case cell body motion was driven by actin polymerization at the front., Thus, computation of force and friction maps suggested that cell body translocation can occur via two different mechanisms: one dependent on actomyosin contraction and involving strong forces and friction, and the other, dependent on actin assembly and associated with low forces and friction. Supported by Swiss National Science Foundation grant 3100A0-112413.

1818/B280

Hijacking the Endothelial Cell Contractile Machinery during Transcellular Invasion by Breast Cancer Cells: a 3D FRET study.

T. Chew[1], S. Khuon[1], R. Dettman[2], P. Sporn[3]; [1]Cell & Molecular Biology, Northwestern University, Chicago, IL, [2]Pediatrics, Northwestern University, Chicago, IL, [3]Medicine, Division of Pulmonary & Critical Care Medicine, Northwestern University, Chicago, IL

To investigate the signals involved in tumor metastasis, we have devised a 3-D matrix system that allows endothelial cells to form vasculature network in vitro. This vascular network is engineered using endothelial cells stably expressing a FRET biosensor for myosin light chain kinase (MLCK) activity (J. Cell Biol. 156: 543-553, 2002). This experimental set-up thus integrates FRET biosensor microscopy and tissue engineering to create a dynamic 3D assay system wherein tumor transendothelial invasion can be studied with very high spatial and temporal resolution from the perspective of the endothelium. The in vitro 3D vasculature network exhibits lumen formation as characterized both by confocal as well as transmission electron microscopy. In situ immunostaining shows that the endothelial cells deposited α4-laminin on the peripheral area of the vessel tube but the lumenal space is relatively cleared of synthesized α4-laminin, indicating that the endothelial cells that form the vasculature undergo polarization, as would normal endothelium under physiological condition. Upon the addition of MDA-MB231 breast cancer cells pre-labeled with CellTracker Red, we frequently observed the invadopodia of the breast cancer cell protruding into the cytoplasm of the endothelial cells (transcellular invasion), rather than through the cell junction (paracellular invasion) as expected. The cytoplasm of the two cells can be easily delineated by confocal microscopy as the endothelial cell and the tumor cell are labeled with different colors. This is consistent with the recently observed transcellular migration of peripheral blood mononuclear cells and MCF cancer cells (Nature Cell Biol. 8: 156-161, 2006; Cell 131:966-979, 2007). Using our MLCK FRET sensor and time-lapse 3D FRET microscopy, we observed a biphasic endothelial signaling response to the interaction with metastatic tumor cells: (1) an initial, general, elevation of MLCK activity above baseline when the cancer cells crawl along the endothelial cell surface, (2) a more acute and marked increase in MLCK activity at the site of tumor entry during active invasion. (Supported by Dept of Defense Breast Cancer Research Program Idea Award W81XWH-06-1-0345)

1819/B281

Protein Domains of Cortactin have Diverse Functions in the Stages of Invadopodium Assembly and Maturation.

M. Oser[1], H. Yamaguchi[3,4], M. Arias[1], R. Eddy[1], V. DesMarias[1], J. van Rheenen[1], J. Condeelis[1,2]; [1]Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, [2]Gruss Lipper Biophotonics Center, Albert Einstein College of Medicine, Bronx, NY, [3]Laboratory of Genome and Biosignal, Tokyo University of Pharmacy and Life Science, Tokyo, Japan, [4]PRESTO, JST, Saitama, Japan

Invadopodia are matrix degrading membrane protrusions in invasive carcinoma cells that are thought to be important for tumor invasion and intravasation. The molecular mechanisms that support the process of invadopodium assembly and maturation are not well understood. Live cell imaging revealed that there is a significant time lag from the formation of the invadopodium precursor structure to the acquisition of matrix degradation activity. Using the protein domains of cortactin to dissect the stages of early invadopodium assembly and maturation, we show that the mechanisms controlling actin polymerization at the invadopodium precursor structure are distinct from the requirements for its initial formation. Specifically, the tyrosine phosphorylation of cortactin is required for actin polymerization at the invadopodium precursor structure, but not for invadopodium precursor formation. We show that the tyrosine phosphorylation of cortactin can control actin polymerization at invadopodium precursors by 2 mechanisms: 1. Regulating the severing activity of cofilin and 2. Localizing Nck1 and N-

WASP activity at invadopodia. In addition, cortactin must be dephosphorylated for stabilization of invadopodia. These findings provide a mechanistic framework for the stages required for the assembly and maturation of the invadopodium precursor structure into a functional matrix degrading invadopodium.

1820/B282

Substrate Dependent Effects of C-Met Inhibitors on HGF-Induced Cell Migration.

B. D. Smith[2], S. C. Wise[2], A. Vogt[3], M. Nakashima[3], R. L. Herber[1], K. I. Hulkower[1]; [1]Platypus Technologies LLC, Madison, WI, [2]Deciphera Pharmaceuticals, LLC, Lawrence, KS, [3]Drug Discovery Institute, University of Pittsburgh, Pittsburgh, PA

The ability of tumor cells to migrate is an essential element in the metastasis of cancer. c-Met is an oncogenic receptor tyrosine kinase (RTK) that is the only known high affinity receptor for hepatocyte growth factor (HGF). The dysregulation of c-Met and HGF in cell migration, invasion and proliferation has been shown to play a role in metastatic cancer progression. We have utilized a recently developed 96-well plate assay for cell migration to study the effects of the c-Met inhibitors PHA-665752 and DP-3590 on PC-3 and A549 cells. Cells were seeded in an annular fashion around a centrally placed silicone stopper within the well which was tissue culture treated or coated with either type I collagen or fibronectin. Cells were then permitted to migrate into the analytic zone formed upon removal of the stoppers, and data was collected by using a plate reader, microscope or Cellomics ArrayScan high content imager. In the presence of 0.5% serum, migration of PC-3 cells was independent of HGF stimulation and refractory to c-Met inhibitors on all substrates tested. However, in the complete absence of serum, PC-3 cell migration was independent of HGF and c-Met on the collagen substrate whereas the cells responded to HGF and c-Met inhibitors on both the tissue culture treated and fibronectin coated surfaces. In contrast, the migration of A549 cells in the presence of 0.5% serum was responsive to HGF stimulation and abolished by both PHA-665752 and DP-3590 on all substrates tested. Latrunculin A, an inhibitor of actin-myosin components of the cytoskeleton, was effective in blocking cellular migration independently of cell line, presence of serum or substrate coating. We conclude that different intracellular signalling mechanisms are utilized during cell migration that are substrate, serum and cell-line dependent. Our findings that the effects of HGF and c-Met on migration can vary depending upon these conditions reveals the need to understand key features of cell-based screening assays used in drug discovery efforts in order to better approximate in vivo, physiologic settings.

1821/B283

KLK13 Overexpression Facilitates Migration of Immortalized Ovarian Surface Epithelial Cells.

N. White, J. Dore; BioMedical Sciences, Memorial University, St. John's, NF, Canada

The human kallikrein-related peptidase family consists of 15 serine proteases located on Ch19q13. Twelve of the fifteen kallikrein-related peptidases are overexpressed in ovarian cancer and there is increasing evidence they may play a role in ovarian carcinogenesis. Kallikrein-related peptidase 13 (KLK13) has been shown to be overexpressed at both the gene and protein levels in epithelial ovarian cancer. KLK13 can cleave major components of extracellular matrix and neutralizing KLK13 antibodies inhibit ovarian cancer cell line migration. Both observations suggest KLK13 functions to increase the metastatic potential of ovarian carcinoma. This study examines the expression of KLK13 in a normal ovarian surface epithelial cell line, as well as ovarian cancer cell lines, to show the involvement of KLK13 in the pathogenesis of ovarian carcinoma. KLK13 mRNA expression was examined with real time PCR in IOSE, CAOV-3, OVCAR-3, and SKOV-3 cell lines. KLK13 mRNA had a 2-fold increase in expression in CAOV-3 and SKOV-3 cells and a 4-fold increase in expression in OVCAR-3 cells when compared to immortalized human ovarian surface epithelial (IOSE) cells. The wild type KLK13, an enzymatically dead KLK13, where an Ala was substituted for Ser218, and a form of KLK13 that cannot be activated, where Gln was substituted for Lys25, were expressed using LNCX2 retroviral infection. Cellular migration was examined by a wound assay in the IOSE cell line by infection with the wild type or mutant KLK13 virus. Analysis of wound healing assays suggest IOSE cells infected with the enzymatically dead KLK13 virus have a slower migration rate than those infected with the wild type KLK13 virus. Also, expression of K25Q mutants decreases migration, acting as a potential dominant negative to the low endogenous levels of KLK13 produced by the IOSE cells. These results suggest the overexpression of KLK13 in ovarian cancer cells plays a role in increasing the metastatic potential of ovarian carcinoma.

1822/B284

SSX Regulate Cancer Invasion via PARP-1, Histone H1 and MMP1 Signaling.

K. Itoh, K. Yoshioka; Biology, Osaka Medical Center for Cancer, Osaka, Japan

The SSX genes were initially identified as fusion partners to the SS18 gene in human synovial sarcomas carrying a recurrent t (X; 18)(p11.2; q11.2) chromosomal translocation. Besides adult human testis, SSX genes were expressed at varying frequencies in a number of malignancies thereby categorized as cancer/testis antigens. We previously reported that SSXs were overexpressed in human soft tissue tumors, and positively correlated with clinical stage. In order to examine the biological function of SSX, we made stable transfectants with wild type SSX using human fibrosarcoma cell line, HT1080, which endogenoulsy expressed SSX1 in high level. The transfectants increased motility and invasiveness using Boyden chamber assay, promoted colony formation in soft agar and lung metastasis in nude mice. By contrast, the lowering of the endogenous expression of SSX1 in HT1080 cells by the treatment with specific siRNA markedly decreased membrane ruffling, chemotaxis, invasiveness and 3D growth in collagen gel but did not affect cell proliferation in the 2D culture. Moreover, SSX1 deficient HT1080 cells showed decreased Rac1-activity and myosin light chain phosphorylation. We are currently evaluating the effect of wrapped liposome containing SSX siRNA in xenograft model. Using oligomicroarray, we found several changes in gene expression profile. Overexpression of SSX increased the expression level and activity of MMP-1, and knockdown of SSX decreased MMP-1 expression. In addition, we identified several binding proteins of SSX, including Histone H1 and PARP-1 using immunoprecipitation and LC/MS/MS. We further found that SSX could bind the promoter region of MMP-1 using ChIP assay. Collectively, these data suggested that SSXs would be novel proteins regulate cancer invasion via PARP-1, Histone H1 and MMP-1 signaling and potential molecular target(s) under clinical setting.

1823/B285

Fibronectin Adhesion Triggers Microtubule Delivery of Abl-Related Gene (Arg) to Regulate Actomyosin Contractility and Focal Adhesion Dynamics in Migrating Cells.

J. Peacock, T. Koleske; Molecular Biophysics and Biochemistry, Yale University, New Haven, CT

In migrating cells, actin polymerization promotes protrusion of the leading edge, while actomyosin contractility powers net cell body translocation. Although they promote F-actin-dependent protrusions of the cell periphery upon adhesion to fibronectin, Abl family kinases inhibit cell migration on fibronectin. We have recently shown that the Abl-related gene (Arg/Abl2) kinase inhibits fibroblast migration by attenuating actomyosin contractility and regulating focal adhesion dynamics (Peacock, Miller, et al, Mol Biol Cell 2007). arg-/- fibroblasts migrate at faster average speeds than wild type cells, while Arg re-expression in these cells slows migration. Surprisingly, the faster migrating arg-/- fibroblasts have more prominent F-actin stress fibers and focal adhesions and exhibit increased actomyosin contractility relative to wild type cells. Interestingly, Arg requires distinct functional domains to inhibit focal adhesions and actomyosin contractility. The kinase domain-containing Arg N-terminal half can act through the RhoA inhibitor p190RhoGAP to attenuate stress fiber formation and cell contractility. However, Arg requires both its kinase activity and its cytoskeleton-binding C-terminal half to fully inhibit focal adhesions. Although focal adhesions do not turn over efficiently in the trailing edge of arg-/- cells, the increased contractility of arg-/- cells tears the adhesions from the substrate, allowing for the faster migration observed in these cells. We have observed that the abilities of Arg to regulate stress fiber formation and focal adhesion dynamics depend on microtubule delivery of Arg to the cellular periphery. A mutant of Arg lacking the microtubule binding domain can no longer attenuate stress fibers nor modulate focal adhesion dynamics. Together, our data strongly suggest that Arg is delivered via microtubules to specific cellular sites in migrating cells, where it plays an important role in regulating cellular contractility and adhesion.

1824/B286

Loss of Profilin-1 Expression Leads to Hypermotile Phenotype of Breast Cancer Cell via Lamellipodial Targeting of Ena/VASP.

Y. Bae[1], L. Zou[1], A. Wells[1,2,3], F. Gertler[4], P. Roy[1,2]; [1]Bioengineering, University of Pittsburgh, Pittsburgh, PA, [2]Pathology, University of Pittsburgh, Pittsburgh, PA, [3]Pittsburgh VA Medical Center, Pittsburgh, PA, [4]Biology, Massachusetts Institute of Technology, Cambridge, MA

Objective: Profilin-1 (Pfn1) expression is significantly down-regulated in various adenocarcinoma including breast cancer. The objective of this study is to determine whether and how loss of Pfn1 expression alters motility of breast cancer cells. Methods: Pfn1 expression was down-regulated in MDA-MB-231 breast cancer cells by siRNA treatment, and the effect of Pfn1 depletion on cell motility was analyzed by time-lapse microscopy. Results: We found that both MDA-MB-231 cells and normal human mammary epithelial cells (HMEC) become more motile when Pfn1 expression is silenced. Surprisingly, kymograph analyses showed that loss of Pfn1 expression actually led to faster membrane protrusion. Both MDA-MB-231 and HMEC showed dramatic enrichment of VASP at their leading edge when Pfn1 expression is downregulated. Inhibition of Ena/VASP proteins by mitochondrial sequestration drastically impaired lamellipodial protrusion and the overall motility of Pfn1-deficient MDA-MB-231 cells, while motility of corresponding control siRNA treated cells were found to be insensitive to VASP inhibition. These data suggest that Ena/VASP proteins not only compensate for the loss of Pfn1's action, but further augment the motility of breast cancer cells in the absence of Pfn1 expression. Interestingly, silencing Pfn1 expression also enhances lamellipodin (Lpd- a PI(3,4)P2-anchoring protein responsible for lamellipodial targeting of Ena/VASP) distribution at the leading edge in a VASP-independent manner. Finally, inhibition of PI3-kinase (important for generation of PI(3,4)P2) delocalizes both VASP and Lpd from the leading edge in Pfn1-depleted cells. This observation is consistent with a possible involvement of Lpd in Pfn1-dependent control of VASP localization. Conclusion: Our findings for the first time highlight a possible mechanism of how reduced expression of a pro-migratory molecule like Pfn1 could actually promote motility of breast cancer cells.

1825/B287

Phosphorylation of Lamellipodin Regulates Ena/VASP Interaction and Is Implicated in Axonal Morphogenesis.

M. Michael, A. Vehlow, C. Navarro, M. Krause; Randall Division of Cell and Molecular Biophysics, London, United Kingdom

Cell motility and axon guidance are essential processes in physiology and disease that involve the coordinated function of actin cytoskeleton regulators. Ena/VASP proteins regulate actin morphology at the leading edge of cells by promoting the formation of elongated actin filaments. Lamellipodin (Lpd), a member of the MRL protein family, is localised to lamellipodia and tips of filopodia. It contains highly conserved Ras association, pleckstrin homology domains and importantly, a series of FPPPP motifs which enable direct interaction and leading edge recruitment of Ena/VASP proteins. Both Ena/VASP and the Lpd *C.elegans* ortholog MIG-10 have been implicated in axon guidance signalling downstream of the netrin receptor DCC. Furthermore, overexpression of Lpd increases lamellipodial protrusion velocity, an Ena/VASP dependent function. Therefore, it has been proposed that Lpd mediates extracellular signals from transmembrane growth factor or guidance receptors to Ena/VASP proteins, thereby regulating their activity. Our findings have revealed that Lpd is phosphorylated and activated downstream of PDGF and netrin signalling pathways which stimulate cell ruffling and axon outgrowth, respectively. We have identified the kinase involved and mapped the phosphorylation sites in Lpd. Phosphorylated Lpd localises to the leading edge. However, we observed that Lpd phosphorylation is not required for its leading edge localisation. Interestingly, we have also uncovered evidence suggesting that Lpd phosphorylation positively regulates its interaction with its main downstream interactor, Ena/VASP proteins. Furthermore, we will present results indicating a role for Lpd in the regulation of axon morphology.

1826/B288

Phosphoinositide Regulation of Proteolytic Post Translational Modification: a Role in Cell Migration.

S. Lal, J. Greenwood; Biochemistry and Biophysics, Oregon State University, Corvallis, OR

Filamin plays an important role in cellular motility by stabilizing the orthogonal networks of actin filaments within the lamella. Although, filamin has been shown to bind phosphoinositides and is cleaved by calpain, the molecular mechanisms regulating filamin dynamics and the turn-over of actin networks in the lamella of migrating cells is not understood. We have found that the binding of phosphatidylinositol

(3,4,5)-triphosphate [PtdIns(3,4,5)-P3] to filamin enhances susceptibility to proteolysis by calpain-2 and a major stable breakdown product migrating at ~250kD is detected. Furthermore, co-sedimentation assays with actin filaments demonstrated that PtdIns(3,4,5)-P3 mediated calpain cleavage results in the loss of actin binding activity of filamin. To understand the mechanism of this functional regulation, the site of cleavage was determined by N-terminal sequencing of the major breakdown product. Consistent with the sedimentation assays, calpain-2 was found to cleave PtdIns(3,4,5)-P3 bound filamin after residue lysine 268 resulting in removal of the actin binding domain. These results suggest that PtdIns(3,4,5)-P3 mediated cleavage by calpain-2 is a potential mechanism for regulating the function of filamin. U87MG glioblastoma cells will be used to study PtdIns(3,4,5)-P3 regulation of filamin proteolysis in migrating cells which have elevated level of PtdIns(3,4,5)-P3 due to mutation in the PTEN gene. When expressed in the U87MG cells, wild type GFP-filamin was found to colocalize with endogenous filamin population as well as with calpain-2 within actin filament networks in the leading edge of migrating cells. In order to determine the influence of filamin cleavage on actin networks, point mutations have been incorporated to produce GFP-filamin proteins resistant to PtdIns(3,4,5)-P3 mediated calpain proteolysis. The results showing expression of calpain resistant filamin mutants will be presented at the meeting. Based on these results, we propose that PtdIns(3,4,5)-P3 regulation of filamin proteolysis by calpain is a potential mechanism controlling turn-over of actin networks at the leading edge of migrating cells.

1827/B289

Actin-Interacting Protein 1 Restores Impaired Chemotaxis of Dictyostelium Na-H Exchanger-Null Cells.

C. Choi[1], h. patel[2], D. Barber[1]; [1]Cell and Tissue Biology, University of California, San Francisco, San Francisco, CA, [2]ECRC-Cancer Biology, University of Edinburgh, Edinburgh, United Kingdom

Emerging evidence indicates that an increase in intracellular pH (pHi) is a necessary signal for efficient directed cell migration. We previously reported that a *Dictyostelium* Na-H exchanger *Dd*NHE1 is necessary for increased pHi during chemotaxis, and that *Ddnhe1*-null (*Ddnhe1*$^-$) cells have impaired chemotaxis, including loss of polarity and attenuated increases in F-actin (Patel and Barber, 2005, JCB). To identify regulators of pH-dependent chemotaxis we developed an assay of plaque formation on heat-killed bacteria to screen for suppression or repression of the *Ddnhe1*$^-$ phenotype using a cDNA over-expression library (provided by D. Robinson, Johns Hopkins University). The screen identified one clone that exacerbated the *Ddnhe1*$^-$ phenotype. The clone encoded for a C-terminal fragment of actin interacting protein 1 (*Dd*Aip1). Stable expression of the fragment, *Dd*Aip1-Δ380, in *Ddnhe1*$^-$ cells further repressed the chemotaxis and F-actin assembly of *Ddnhe1*$^-$ cells in response to cAMP. In contrast, stable expression of full-length *Dd*Aip1 rescued impaired chemotaxis of *Ddnhe1*$^-$ and enhances F-actin assembly. Expression of *Dd*Aip1-full length or -Δ380 had no effect on chemotaxis or F-actin assembly in Ax2 cells. Aip1 functions to promote cofilin-regulated F-actin dynamics and cofilin activity is recognized to be pH-dependent. Aip1 interacts with cofilin via its N-terminus while its C-terminus caps F-actin independent of its interaction with cofilin. We confirmed direct binding of purified full-length Aip1 to cofilin and found that the interaction is pH-insensitive. We predict that *Dd*Aip1-Δ380 represses *Ddnhe1*$^-$ chemotaxis because it retains capping activity but not cofilin binding and cofilin-regulated F-actin assembly. However, full-length Aip1 restores chemotaxis because it promotes cofilin-induced increases in F-actin, which is reduced in *Ddnhe1*$^-$ cells. These data further suggest a role for pH-dependent cofilin activity in actin dynamics and chemotaxis and identify Aip1 is an important regulator of this mechanism in *Dictyostelium* cells.

1828/B290

Dynamic, Actin-Rich, Arp2/3-Dependent Structures Form on Specific Cells during C. elegans Gastrulation.

M. Roh, B. Goldstein; University of North Carolina - Chapel Hill, Chapel Hill, NC

Morphogenesis requires regulated cell movements and intricate coordination of cytoskeletal dynamics. In C. elegans, the first morphogenetic movement, gastrulation, is initiated by the internalization of two endodermal precursor cells (E cells). The current model for how these cells become internalized is that the E cells develop an apicobasally-polarized distribution of non-muscle myosin II, which becomes activated by regulatory light chain phosphorylation, causing the E cells to apically constrict. This has been hypothesized to pull a ring of six neighbouring cells that meet in the gap left by the inward movement of the E cells. Severson et al. (2002) discovered that depleting the Arp2/3 complex in C. elegans does not arrest cell divisions but does result in gastrulation defects. Arp2/3 is known to function in morphogenesis in various developmental systems, but its specific roles during morphogenesis are not well understood. We have found that in Arp2/3 depleted C. elegans embryos, although the E cells do not fully ingress, the E cells are polarized normally, and myosin accumulates and becomes activated in the apical region of the E cells. We found that half of the ring of six neighbouring cells (three of the six cells) extend short, dynamic, F-actin-rich structures near their apical borders with the E cells, where cell flattening has previously been reported by SEM. These cells have been hypothesized previously to roll in their direction of movement; we are currently testing this. These F-actin-rich structures do not form after Arp2/3 depletion. We are currently driving Arp2/3 expression in specific cells to assess whether Arp2/3-dependent functions are required only in specific cells for gastrulation. These results suggest a new layer to our existing model of C. elegans gastrulation: In addition to apical constriction, internalization of the endoderm may involve dynamic, Arp2/3-dependent, F-actin-rich extensions on specific neighbouring cells.

1829/B291

Major Sperm Protein Phosphorylation and Its Putative Role as an Intracellular Signaling Molecule during Spermatid Activation in Caenorhabditis Elegans.

J. Fraire-Zamora, R. A. Cardullo; Biology, UC Riverside, Riverside, CA

Nematode spermatozoa are highly specialized cells that are aflagellate and extend a pseudopod to initiate motility. Unlike other amoeboid cells, the cytoskeletal elements that are responsible for pseudopod formation are not actin-based microfilaments but are composed of a unique protein known as the Major Sperm Protein (MSP). MSP constitutes 40% of the total soluble proteins in the sperm cell and, in addition to its role in pseudopod extension, has also been implicated in oocyte maturation and ovulation. However, to date, there is no evidence of direct involvement of MSP in intracellular signaling pathways that lead to pseudopod extension. Additionally, no direct phosphorylation of MSP has been demonstrated in nematode sperm. Here, we show evidence for MSP phosphorylation in *C. elegans* spermatozoa using immunoblot

detection coupled to Mass Spectrometry identification. Analysis of the resulting sequences predict putative phosphorylation sites suggesting a direct role of MSP as an intracellular signaling protein involved in the cytoskeletal dynamics during sperm activation leading to pseudopod extension.

1830/B292

Cryo-Electron Tomography of MSP Filaments Assembled in Extracts of Ascaris Sperm.

A. Malowney[1], M. Stewart[2], T. M. Roberts[1]; [1]Biological Science, Florida State University, Tallahassee, FL, [2]MRC Laboratory of Molecular Biology, Cambridge, United Kingdom

Localized assembly of the MSP-based cytoskeleton powers lamellipodial protrusion in the amoeboid sperm of *Ascaris suum* in a manner analogous to actin-based cell migration. MSP may be induced to polymerize *in vitro* by addition of ethanol or other water-miscible alcohols to purified MSP (King et al. 1992. JSC 101:847) or by the addition of ATP to *Ascaris* sperm cell extracts. ATP-induced MSP polymerization in cell-free extracts closely resembles the mode of polymerization observed *In Vivo* and is thus the preferred model for characterizing specific protein interactions. Here we seek to determine the specific interactions between MSP and its accessory proteins responsible for MSP filament polymerization. Addition of ATP to the S100 fraction of sperm extracts treated with triton x-100 allows for a cell-free analysis of MSP polymerization via observation of isolated filaments which are readily detectable by electron microscopy. We analyzed isolated filaments using cryo-electron tomography to determine native filament structure and to detect variable filament assembly patterns mediated by associated proteins. Addition of the putative MSP filament capping protein MFP1 or polymerization-promoting MFP2 have antagonistic effects on MSP polymerization *in vitro* (Buttery et al. 2003. MBC 14:5082), and here we seek to characterize their effects on cell-free polymerization at the structural level. Supported by NIH Grant R37 GM29994.

1831/B293

Functional Significance of the Golgi-Derived Microtubule Array in Motile Cells.

P. M. Miller, N. Efimova, A. R. Maia, I. Kaverina; Cell & Developmental Biology, Vanderbilt University Medical Center, Nashville, TN

Our lab has recently shown that microtubules can be nucleated at the Golgi apparatus in CLASP-dependent manner. However, the functional significance of Golgi-derived microtubule array remains unclear. Now we show that microtubules that emanate from the Golgi play an important role in Golgi ribbon assembly as well as vesicular trafficking toward the leading edge of motile cells. Utilizing live cell imaging, we have found that Golgi-derived microtubules are required for the initial step in the two-step process of Golgi ribbon assembly. This two-step sequence was observed for post-mitotic Golgi assembly as well as for experimental conditions of microtubule re-growth after nocodazole washout. In the first step, Golgi mini-stacks are gathered into clusters throughout the cytosol. In the second step, these clusters are transported toward the cell center along centrosomal microtubules to complete Golgi ribbon assembly. Both steps require minus end-directed motor dynein activity. Abolition of the Golgi-derived microtubule array via siRNA-driven CLASP depletion eliminates first step of assembly. This leads to defects in Golgi cisternae fusion and overall Golgi ribbon organization. Interestingly, polarized trafficking toward the leading edge of CLASP-depleted cells is also disrupted. Trafficking defects correlate with inability of these cells to establish and sustain directional migration patterns. Based on these results, we suggest that CLASP-dependent microtubules are essential for proper Golgi organization and, subsequently, for polarized post-Golgi trafficking and directional cell migration.

Cytoskeletal Organization II (1832 – 1845)

1832/B294

The Mitotic Kinesin Ncd Exhibits Directional Microtubule Sliding.

G. H. Fink[1], L. Hajdo[2], K. Skowronek[2], C. Reuther[1], A. A. Kasprzak[2], S. Diez[1]; [1]MPI-CBG, Dresden, Germany, [2]Nencki Institute of Experimantal Biology, Warsaw, Poland

During mitosis and meiosis, the bipolar spindle mediates chromosome segregation through a combination of microtubule sliding as well as microtubule growth and shrinkage. One of the motors involved, kinesin-14, causes spindle collapse in the absence of kinesin-5, participates in spindle assembly, and modulates spindle length. However, the molecular mechanisms underlying these activities are not known. Here, we report that Drosophila melanogaster kinesin-14 (Ncd) alone crosslinks microtubules, sliding apart anti-parallel ones but locking together parallel ones. Using single molecule imaging we show that Ncd diffuses along microtubules in a tail-dependent manner and switches its orientation between sliding microtubules. Taken together, these results show that kinesin-14 causes sliding and expansion of an anti-parallel microtubule array by dynamic interactions through the motor domain on the one side and the tail domain on the other. Such mechanism accounts for the roles of kinesin-14 in spindle organization.

1833/B295

The Microtubule Network in Both Polarized and Unpolarized MDCK Epithelial Cells Contains Distinct Populations of Acetylated Microtubules and Detyrosinated Microtubules That Differ in Localization, Stability and Regulation.

L. Ligon[1,2], G. Quinones[1,2], M. Salt[1,2], S. Tulin[1,2]; [1]Biology, Rensselaer Polytechnic Institute, Troy, NY, [2]Center for Biotechnology and Interdisciplinary Studies, Rensselaer Polytechnic Institute, Troy, NY

Microtubules can be modified post-translationally in several ways, and it is thought that these modifications (PTMs) may mark individual filaments for specific functions or targets. Little is known, however, about the regulation of microtubule PTMs or about the functions of specific PTMs (or combinations thereof). To address these questions, we have been examining the microtubule network in MDCK cells before and after polarization. Unpolarized cells have both acetylated microtubules (acet-MT) and detyrosinated microtubules (glu-MT), but while there is some overlap, these PTMs mark distinct populations of microtubules. In polarized cells, this difference is even more striking as the glu-MTs are primarily localized to the basal domain of the cell and the acet-MTs are mainly found in the mid- and apical domains. The ratio of glu-tubulin to total tubulin decreases significantly as cells undergo polarization, but increases again after polarization. The ratio of

acet-tubulin to total tubulin, on the other hand, undergoes a more gradual decrease with polarization. In unpolarized cells, acet-MTs are initially more sensitive to nocodazole-induced depolymerization than glu-MTs, but in polarized cells, the glu-MTs are more sensitive to nocodazole-induced depolymerization. Finally, glu-MTs appear to be more sensitive to disruptions of the Rho GTPase than acet-MTs in both unpolarized and polarized cells. Together these data suggest that the acetylation PTM and the detyrosination PTM mark distinct populations of microtubules, which differ in their localization, stability and regulation by GTPases. The microtubule cytoskeleton in epithelial cells undergoes a complete reorganization with the establishment of polarity, and it is possible that these differential populations of microtubules may play a role in this restructuring.

1834/B296

Microtubule Severing Activity Scales Spindle Length in Xenopus Egg Extract.

R. Loughlin[1], J. Wilbur[2], F. Nedelec[3], R. Heald[2]; [1]Biophysics Graduate Group, University of California, Berkeley, Berkeley, CA, [2]Molecular and Cellular Biology, University of California, Berkeley, Berkeley, CA, [3]Cell Biology and Biophysics, European Molecular Biology Laboratory, Heidelberg, Germany

The physiological determinants of spindle size are not understood. To elucidate mechanisms of mitotic spindle assembly and scaling, we developed a computational model for spindle formation based on microtubule (MT) dynamics and the activities of three conserved MT-based motor proteins. In our model, we found that focusing of MT minus ends at spindle poles depended not only on motor-driven movements, but also required localized MT depolymerization at the poles, and that altering this parameter changed spindle length. To test whether this mechanism might be responsible for differential scaling of larger mitotic spindles in X.laevis compared to the smaller spindles in X. tropicalis egg extracts, we evaluated the stability of fluorescently-labeled, Taxol-stabilized MTs in extracts using a quantitative flow cell assay. We found that katanin-dependent MT severing, but not kinesin-13 dependent MT depolymerization, was significantly greater in X. tropicalis extracts. Extract mixing experiments revealed that, like spindle length, severing activity was titratable and dose-dependent in the two extracts. Katanin inhibition by antibody addition increased spindle length in both extracts, and, as expected, had a much more dramatic effect on the smaller X. tropicalis spindles. However, katanin protein levels as determined by Western blot and spindle pole localization in immunofluorescence were similar in both extracts. Current efforts with partially purified katanin aim to test whether altering katanin levels scales spindle length.

1835/B297

Microtubule Protrusions Are Regulated by the Antagonism of Tubulin and Actin Filament Systems in Breast Tumor Cells.

E. Balzer[1,3], R. Whipple-Bettes[2,3], E. Cho[1,3], M. A. Matrone[1,3], S. S. Martin[3,2,1]; [1]Molecular Medicine, University of Maryland, School of Medicine, Baltimore, MD, [2]Physiology, University of Maryland, School of Medicine, Baltimore, MD, [3]Stewart and Marlene Greenebaum Cancer Center, University of Maryland, School of Medicine, Baltimore, MD

Breast tumor metastasis depends on cytoskeletal alterations like those of the epithelial-mesenchymal transition (EMT), which is characterized in part by the acquisition of dynamic cortical actin structure. We have described highly dynamic tubulin-based protrusions of the plasma membrane, which are observed at high frequency in tumor cell lines of great metastatic potential. These tubulin microtentacles (McTN) are infrequent in untransformed cells and tumor lines of low metastatic potential. Actin depolymerization enhances McTN formation, thus McTNs are structurally distinct from filopodia, and other microfilament processes. Jasplakinolide (jas) forces microfilament disorder in vitro, increasing McTN frequency in all cell lines tested without reduced cell viability. Propidium iodide was excluded from cells with active McTNs, suggesting that jas-induced protrusions are produced by viable cells. Immunofluorescent labeling revealed that jas induces organization of α-tubulin into outwardly projecting filaments, extending from the cell periphery concomitant with the collapse of filamentous actin. Microtubule (MT) stabilization augmented this response and increased McTN frequency in suspended cells. Aggregation assays demonstrated that cells coalesced into large suspended colonies more rapidly than their untreated counterparts in a manner consistent with McTN induction. These results describe a novel antagonism between actin and tubulin cytoskeletal networks in the context of membrane protrusions; structures of potential relevance for endothelial binding and extravasation of disseminated tumor cells. Our findings advise caution regarding the application of MT-stabilizing agents to transformed cells of inherent actin disorder.

1837/B299

Functional Characterization of a Novel Microtubule-Actin Crosslinking Protein, MACT1.

M. J. Stroud, J. D. Humphries, C. Ballestrem, R. A. Kammerer; Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom

The microtubule (MT) and actin cytoskeletons are fundamental to cell integrity, as they control a host of cellular activities. Until recently, studies have investigated these cytoskeletal components as separate entities; however, it has become increasingly clear that the microtubule and actin cytoskeletons function in an interdependent way. Elucidating how the two components interact will be key to our further understanding of fundamental cellular processes, such as cell division, growth, polarization and migration. Here, we have characterized a novel protein, named MACT1, which contains a putative actin binding calponin homology domain and a putative MT binding GAS2-related domain. The identified protein predominantly localizes to MTs, and shows evidence of actin localization. We identified that the C-terminus of the protein is involved in binding to the MT plus end binding protein 1 (EB1). When co-expressed with EB1, full-length MACT1 recruitment to MTs is enhanced, whilst C-terminus deficient MACT1 localizes predominantly to the actin cytoskeleton. Complementary to this immunofluorescence data, we show that the C-terminus of MACT1 interacts with EB1 in vitro with a high binding affinity.

1838/B300

Microtubule Function and Dynamics during HGF-Induced Tubulogenesis in 3D Culture.

S. Gierke, T. Wittmann; Cell and Tissue Biology, University of California San Francisco, San Francisco, CA

Apical/basal polarized epithelial cells transition to a migratory phenotype during many developmental processes and during the metastatic transformation of tumor cells. This involves loss of polarity and changes in cell shape, which are ultimately controlled by spatiotemporal

regulation of cytoskeletal dynamics. Although dynamic microtubules (MTs) are required for cell migration in 2D, MT function and dynamics has not been examined during cell shape changes or migration in more physiological 3D environments. To address this, we adapted a well established 3D epithelial morphogenesis model for high resolution microscopy. Madin Darby canine kidney (MDCK) cells embedded in extracellular matrix (ECM) form spherical cysts with distinct apical/basal polarity. When treated with Hepatocyte Growth Factor (HGF), these cysts undergo tubulogenesis during which cells lose polarity and migrate into the ECM. We hypothesize that spatiotemporal MT regulation is important for this process, and our initial objective was to characterize changes in MT organization and dynamics during early stages of HGF-induced tubulogenesis. Prior to HGF addition, cells had a dense MT array at the apical surface, sparse MTs at the basal surface and lateral MT bundles parallel to the apical/basal axis. After 24 hours in HGF, many cells had formed long extensions protruding from their basal surface that contained parallel bundles of MTs. Live cell imaging of EB1-EGFP, revealed a large number of growing MT ends, and MTs growing all the way to the tip of HGF-induced extensions often hit and physically deformed the membrane. We tracked EB1-EGFP comets to estimate MT growth rates, and found that MTs in extensions displayed an increased growth rate and persistency as compared to unstimulated cysts. Even in cells having extensions that exceeded the cell diameter several fold, the centrosome remained near the apical surface indicating that centrosome reorientation is not required for migratory cell polarity in this system. Together these data show that it is possible to image cytoskeletal dynamics in 3D culture at high spatial and temporal resolution and that MTs undergo dynamic changes during HGF-induced tubulogenesis that are different from 2D.

1839/B301

Putative B″ Subunit of Protein Phosphatase 2A TON2 Localizes at the Preprophase Band Microtubule Arrays and Regulates Dynamic Properties of Microtubules in Arabidopsis.

V. Kirik[1], C. Metzinger[1,2], D. Ehrhardt[1]; [1]Plant Biology, Carnegie Institution, Stanford, CA, [2]Department of Biological Sciences, Stanford University, Stanford, CA

The preprophase band (PPB) forms by the global rearrangement of cortical microtubules into an equatorial ring during the transition between G2 and M phases of the cell cycle. This transient structure plays a crucial role in cell division by providing a spatial cue for cell plate positioning at cytokinesis. To study molecular mechanisms regulating PPB formation we analyzed cellular functions of the Arabidopsis TON2 protein - a putative B″ subunit of protein phosphatase 2A, which is required for PPB formation. We created Arabidopsis plants expressing YFP:TON2 fusion protein and found that TON2 is associated with the PPB, suggesting a direct role of TON2 in PPB formation. To address the role of TON2 in the microtubule cytoskeleton we introduced a YFP:TUA marker into the *ton2* mutant and analyzed microtubule behaviors. We found that microtubule dynamic properties and nucleation are regulated by TON2. In conclusion our results suggest that localized activity of TON2 in regulation of microtubule dynamics and/or nucleation is important for the PPB formation. Currently we are investigating which of the microtubule properties under TON2 regulation are essential for the PPB formation.

1840/B302

Electron Tomography Reveals the Subpellicular Microtubule Cytoskeleton Architecture of Trypanosoma Brucei.

J. L. Hoog[1], S. Lacomble[1], R. J. McIntosh[2], A. Hoenger[2], K. Gull[1]; [1]University of Oxford, Oxford, United Kingdom, [2]Department of MCD Biology, University of Colorado, Boulder, CO

The African trypanosomes are unicellular parasites that cause devastating disease: sleeping sickness in humans and Ngana in cattle. Trypanosomes vary in shape during the cell cycle as well as in the different life cycle forms found in the mammalian host and the tsetse fly vector. Cell shape is essential for pathogenicity and is precisely maintained by a corset of microtubules, which are cross linked to each other and the inner surface of the plasma membrane. We are undertaking a large scale electron tomography investigation to visualize the ultrastructural rearrangements of this cytoskeleton, both as it changes during the cell cycle and between its different life forms. To ensure best possible morphology, we developed a method to high pressure freeze and freeze substitute trypanosomes, using a fast protocol using only 2% uranyl acetate for one hour in the freeze substitution step. Tomography and other EM techniques reveal specific stop and start sites between the microtubules of the array. We have identified a number of MAPs including a *T. brucei* homologue of the microtubule end-binding protein 1 (EB1), which localizes to the posterior end of the cell where the majority of microtubule plus ends are found.

1841/B303

Ultrastructure of Ctenophore Tissues Fixed by High-Pressure Freezing Followed by Freeze-Substitution.

A. Moss[1], T. Mueller-Reichert[2]; [1]Biological Sciences, Auburn University, Auburn, AL, [2]EM Technology Development, Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany

Ultrastructural analyses of marine tissues from gelatinous organisms are notoriously difficult because of the typically poor quality of chemical fixation. The best fixation methods for ctenophores have used a combination fix (J. Cell Biol. 89:495). In selected rare cases very simple glutaraldehyde fixation can produce very good fixation (Zoomorph. 107:319). Detailed understanding of the cellular architecture, coupled with functional knowledge of the associated processes and improved molecular data, are critical for better understanding of these organisms, which appear to be the direct descendents of the common ancestor to all animals (Nature 452:745). Here we show that high-pressure freezing results in the excellent preservation of fine structure in these particularly watery tissues (98% water). Comb plates, tentacles and associated epithelia of *Pleurobrachia pileus* and *Euplokamis dunlapae* were cryoimmobilized with an EM PACT2+RTS (Leica). Tissues were freeze-substituted in acetone containing 1% osmium tetroxide and 0.1% uranyl acetate, and thin-layer embedded in Epon/Araldite. Our results confirm the presence of striated muscle in the tentillae of *E. dunlapae* and reveal exceptional detail in the microtubule and microfilament cytoskeleton. We observed directly that clear-core vesicles of the presynaptic boutons were attached to microfilaments. Comb plate cilia and 3-8 bridges were rendered in considerable detail, and previously unobserved dense core extracellular microvesicles were observed at ciliary bases; basal bodies showed greatly improved ultrastructure and microtubule arrays. Nuclear envelopes were uniform with precise organization of the pores. RER was faithfully rendered, with numerous clustered ribosomes evident on the surface. Giant ciliary rootlets (Hydrobiologia 530:145) showed improved preservation. We conclude that this is a vastly improved, reliable method to yield greatly

improved fixation of ctenophores tissues. Support: NSF MCB0348327, a MBL Summer Fellowship (AGM, TMR), and AL-EPSCoR:NSF EPS-0447675.

1842/B304

Dual Activity of KIF17 at MT Plus-Ends and the Cortex Regulates Microtubule Stabilization in Epithelial Cells.

F. Jaulin, G. Kreitzer; Cell and Developmental Biology, WEill Medical College of Cornell University, New York, NY

MT capture, stabilization and reorganization are essential to development of asymmetry in numerous cell types. An evolutionarily conserved process, known as MT-search-and-capture, promotes MT reorganization associated with polarization in yeast and fibroblasts. During epithelial polarization, a subset of microtubules (MTs) is stabilized and reorganized but the mechanism underlying these events remains unclear. We show that two essential effectors of search and capture, the MT plus-end binding protein EB1 and the membrane-cytoskeletal scaffold IQGAP1, participate in MT stabilization in epithelial cells. We also provide the first evidence in metazoa that, like in yeast, these events require an anterograde, MT plus-end associated kinesin, KIF17. Overexpression of active KIF17 resulted in increased MT stability in epithelial cells. By contrast depletion of KIF17 by shRNA decreased stable MTs. KIF17 associates dynamically with MT plus-ends in an EB1 dependent manner. KIF17 also localizes to the cell cortex where it interacts with IQGAP1. KIF17-mediated MT stabilization involves (i) attenuation of MT polymerization rates that is modulated by EB1 and (ii) MT capture mediated by IQGAP1. We propose that MT-search-and-capture controls MT stabilization associated with epithelial cell polarization and that KIF17, together with EB1 and IQGAP1, play a critical function in this mechanism.

1843/B305

Post-Translational Modification of Sep7 and Regulation of Septin Organization.

R. A. Meseroll, B. DeMay, A. S. Gladfelter; Department of Biological Sciences, Dartmouth College, Hanover, NH

Septins are highly conserved proteins that function in diverse cellular processes, including cytokinesis, morphogenesis, and exocytosis. Individual septin proteins assemble into hetero-oligomeric complexes, which further assemble into nonpolar filaments. These filaments complex to form higher order structures, such as rings and bundled filaments. While recent structural and supra-structural analyses *in vitro* have revealed the composition and arrangement of individual septin filaments, the mechanisms of septin assembly into the higher order structures seen *In Vivo* are not well understood. Diverse higher-order septin structures form in the filamentous fungus, *Ashbya gossypii*. *A. gossypii* has five septins (Cdc3, Cdc10, Cdc11, Cdc12, and Sep7) that assemble into structures at hyphal tips, hyphal bases, and at sites of incipient hyphal growth, in addition to forming rings throughout the cell body. These structures are morphologically distinct, despite lying in close proximity on the cortex of a common cytoplasm. Not only do these septin rings differ in appearance, but mutants of different regulators perturb a subset of structures. We have shown that the deletion of *ELM1*, which encodes a kinase, leads to a loss of only a subset of septin rings while other rings appear normal. In contrast, disruption of Sep7p leads to a loss of only branch-localized septin structures, indicating that different ring morphologies may depend upon the differential regulation of individual septin subunits. We hypothesize that localized post-translational modifications are responsible for assembly and/or maintenance of specific higher order structures. We show here that *A. gossypii* Sep7p is phosphorylated, and may receive additional post-translational modifications, which plays a role in higher-order septin structures. We are currently correlating the phosphorylation status of Sep7p with ring morphology. With this work, we will learn how modification of specific septin subunits leads to the different higher order structures that are observed in cells.

1844/B306

Assembly and Maturation of the Septin Cortex in a Filamentous Fungus: Rules for In Vivo Septin Organization.

B. DeMay, P. Occhipinti, A. S. Gladfelter; Biological Sciences, Dartmouth College, Hanover, NH

Septins are conserved, GTP-binding proteins that assemble into diverse higher-order structures in cells including filaments, rings, and gauzes with varied cellular functions. Purified septins form symmetrical or apolar rods of heter-oligomers in which the septin subunits are in a linear array. *In vitro*, the rods are linked end to end to form extended, non-polar filaments that are frequently paired. Very little is known in any system about how the septin filaments formed *in vitro* relate to higher-order septin structures observed by either light or electron microscopy *In Vivo*. We have developed a filamentous fungus called *Ashbya gossypii* as a model system for analysis of the *In Vivo* rules of septin organization. This system has the benefits of molecular genetics available in budding yeast but displays unique higher-order septin structures that persist for many hours, similar to those found on the cortex of mammalian neuronal cells. Using 4D, quantitative fluorescence microscopy of cells expressing Sep7-GFP from the endogenous locus, we show that septins assemble into morphologically distinct classes of rings that vary in dimensions, intensity, duration and positions across developing fungal mycelia. Notably, these diverse structures can assemble and co-exist within close proximity of each other on the cortex of a common cytoplasm. We demonstrate that new septin proteins are added with time to assembled rings, indicating that septin filaments polymerize within pre-formed rings or that individual subunits can be incorporated into mature rings. Septin ring maintenance does not require the actin or microtubule cytoskeletons however F-actin cables are necessary for de novo assembly of new septin filaments in the cytosol and the genesis of higher-order structures on the cortex. Differences in higher-order structures are not due to the presence or absence of different septin subunits but rather are due to the specific activity of regulatory factors. Strains lacking the kinases Elm1p and Gin4p and the GAPs for Cdc42p show loss of only a subset of the septin structures. Therefore, we show that distinct higher-order septin structures are under control of discrete signaling pathways.

1845/B307

Involvement of Microtubules in the Repolarisation Process of Cells Subjected to Mechanical Force.

A. M. Goldyn[1], B. Aragués Rioja[1], J. P. Spatz[1], c. ballestrem[2], R. Kemkemer[1]; [1]New Materials and Biosystems, Max Planck Institute for Metals Research, Stuttgart, Germany, [2]Welcome Trust Centre for Cell-Matrix Research, University of Manchester, Manchester, United Kingdom

Mechanical stress induces adaptive remodelling of the actin cytoskeleton to yield changes in cell shape and polarisation. We investigated the role of microtubules during polarity events induced by directional cyclic stretching. Application of such force lead to the polarisation of cells

perpendicular to the stretch axis and involved a reorganisation of focal adhesions, the actin cytoskeleton, and microtubules. We showed that microtubules are essential for force-induced migration but dispensable for polarised reorientation of cells, cell-matrix adhesions and the actin cytoskeleton. The microtubule orientation indicated that their stretch-induced reorganisation depended entirely on actin filaments. In contrast to directional stretch-induced cell migration, neither stabilisation nor disruption of microtubules affected the repolarisation of cells, its actin cytoskeleton and focal adhesions. The activity of Rho GTPases controls cell movements and polarity. We studied whether stretching affected the Rho GTPase activity using FRET and ELISAs, and tested their correlation to microtubules. Whereas RhoA activity levels increased by stretching, Rac1 activity levels remained constant showing high local levels in protrusions. This Rac1 activity gradient was unchanged in absence of microtubules. Our results demonstrate that focal adhesions together with the actin cytoskeleton coordinate cell polarity. Since even stabilised microtubules follow the reorientation of the actin network we speculate that proteins involved in crosslinking or guidance of microtubules with or along filamentous actin could facilitate the microtubular reorientation. The sensing of forces by the cell's adhesion machinery as well as its molecular reorganisation in response to stretching forces remains functional without microtubules. This suggests that the reported targeting of focal adhesions by microtubules, which is thought to be essential for focal adhesion turnover during cell migration, is not needed for the enormous stretch-induced reorganisation of adhesion sites and the actin cytoskeleton. Thus the molecular cues involved in the force-induced reorientation of cells are distinct from those required for coordinated cell migration.

Microtubule Dynamics and Assembly (1846 – 1872)

1846/B308

Probing the Roles of Centrosomal Microtubule Nucleation Factors in Anastral Spindle Assembly.

T. J. Maresca[1,3], J. Gatlin[1,3], E. D. Salmon[1,3], T. Mitchison[1,2], A. Groen[1,2]; [1]Cell Division Group, Marine Biological Laboratory, Woods Hole, MA, [2]Systems Biology, Harvard Medical School, Boston, MA, [3]Biology, University of North Carolina, Chapel Hill, NC

The nucleation of microtubules is required for spindle assembly during cell division. Microtubules assemble/nucleate from centrosomes and chromatin during mitosis in most cell types. We compared the relative contributions of centrosome-mediated microtubule assembly factors (such as gamma-tubulin and XMAP215) to chromatin-mediated microtubule assembly factors (such as TPX2) in regulating the formation of anastral spindles assembled in X. laevis egg extracts. Neither XMAP215 nor gamma-tubulin depleted extracts assembled bipolar spindles; however, we found that these defects were rescued by addition of XMAP215 (2-3X endogenous concentration), but not by either TPX2, EB1 or functionally perturbing MCAK antibody. Interestingly, the spindle assembly defects of TPX2 (a chromatin-dependent microtubule assembly factor) depleted extracts could be rescued by the addition of purified TPX2, EB1 or XMAP215. Thus, gamma-tubulin and XMAP215 exhibit functional redundancy and play a role that is distinct from the activity of TPX2 in microtubule nucleation during anastral spindle assembly. Finally, we propose a model for anastral spindle assembly whereby gamma-tubulin and XMAP215 uniformly nucleate microtubules throughout the cytoplasm that become organized into a spindle around chromosomes due to localized signals that activate growth/rescue factors and inhibit catastrophe factors.

1847/B309

Microtubule Growth in an Anastral Spindle from Both the Chromosomes and Poles.

Z. Liang, M. Hallen, S. Endow; Cell Biology, Duke University, Durham, NC

Anastral spindles lack centrosomes and assemble in an unusual manner that involves de novo formation of foci or asters and microtubule nucleation from the chromosomes. Microtubule organization in anastral spindles is also unusual—long and short microtubules are randomly interspersed and minus ends are present throughout the spindle. Microtubule dynamics in anastral spindles is poorly understood. Here we analyze microtubule growth in the Drosophila oocyte meiosis I spindle using a plus-end tracking protein, EB1, fused to a bright GFP. We find EB1 particles present throughout the spindle, presumably binding to growing microtubule plus ends. Unexpectedly, the EB1 particles move both towards the spindle poles and towards the chromosomes at the equator. By particle tracking and fluorescence flow analysis, we show that the velocity of movement, corresponding to microtubule growth rates, is the same in each direction, but slower than growth from the poles in early mitotic spindles. Turnover of EB1 and α-tubulin in FRAP assays occurs at similar rates at the oocyte meiosis I spindle equator and poles, in contrast to mitotic spindles, where turnover is much faster at the poles than equator. Fluorescence loss in photobleaching (FLIP) assays show EB1 and α-tubulin transport or movement at comparable rates from the oocyte meiosis I spindle poles to the equator and from the equator to the poles. This differs from early mitotic spindles where movement of EB1 and α-tubulin is much faster from the poles to the equator, consistent with microtubule growth from the poles towards the chromosomes. Our findings compel the conclusion that microtubules grow both from the chromosomes towards the poles and from the poles towards the chromosomes in the mature oocyte meiosis I spindle. This differs strikingly from early mitotic spindles, where microtubule growth is predominantly from the poles towards the chromosomes. It implies that microtubules in the anastral oocyte spindle are of mixed polarity, rather than oriented with minus ends at the poles as in mitotic spindles. This indicates a fundamental difference between anastral and astral spindles, which is likely to be important in understanding anastral spindle mechanics and function.

1848/B310

Effects of Tau Pseudophosphorylation at Residues Thr231, Ser262, Ser396, and Ser404 upon Microtubule Assembly and the Regulation of Microtubule Dynamic Instability.

E. Kiris[1,2,3], D. Ventimiglia[1,2], M. R. Gaylord[1,2], A. Altinok[3,4], E. Sargin[3,4], K. Rose[3,4], B. S. Manjunath[3,4], M. Jordan[1,2], L. Wilson[1,2,3], S. Feinstein[1,2,3]; [1]Dept. of Molecular, Cellular and Developmental Biology, Univ. of California Santa Barbara, Santa Barbara, CA, [2]Neuroscience Research Institute, Santa Barbara, CA, [3]Center for BioImage Informatics, Santa Barbara, CA, [4]Dept. of Electrical and Computer Engineering, Univ. of California, Santa Barbara, Santa Barbara, CA

Tau is a neural specific microtubule-associated protein that is crucial for the proper development and maintenance of the nervous system. Mechanistically, tau binds directly to microtubules, promotes microtubule assembly and regulates microtubule dynamics. Although tau is

necessary for normal development and maintenance of the nervous system, abnormal tau behavior has long been associated with numerous neurodegenerative diseases, including Alzheimers and related dementias. Biochemically, a large body of data implicates abnormal tau phosphorylation as a key to pathological tau action. Historically, most work on tau phosphorylation has assessed the effects of single phosphorylation events; however, in vivo, tau molecules are multiply phosphorylated. One major question being tested is whether or not the combinatorial effect of multiple phosphorylation events is the sum of the effects of the individual phosphorylation events. We are also seeking to better understand the roles of particular defined phosphorylation events upon tau's mechanisms of action. Specifically, we are assessing the mechanistic effects of single and double phosphorylation events at four key amino acid sites (thr231, ser262, ser396 and ser404) upon the ability of tau molecules to assemble microtubules as well as to regulate their dynamics. For technical reasons, we have used 'pseudophosphorylation' rather than enzymatic phosphorylation to achieve site-specific 'phosphorylation'. Key observations include that phosphorylation of tau does influence regulation of microtubule dynamics and that the combinatorial effect of two pseudophosphorylation events is not necessarily the additive effect of the two individual events. Given that the ratio of 3-repeat to 4-repeat tau is critical for the development and maintenance of the nervous system, we also sought to assess mechanistic differences between pseudophosphorylated 3-repeat and 4-repeat tau. Indeed, several examples were identified in which pseudophosphorylation at the same sites yielded different and occasionally opposite regulatory effects upon microtubule dynamics in 3-repeat versus 4-repeat tau.

1849/B311

Artificial Mitotic Spindles Assembled Using Dielectrophoresis and UV Protein Patterning Support Complex Kinesin-Driven Dynamics.

M. Uppalapati[1], Y. Huang[2], V. Aravamuthan[1], T. N. Jackson[2], W. Hancock[1]; [1]Bioengineering, Pennsylvania State University, University Park, PA, [2]Electrical Engineering, Penn State University, University Park, PA

Mitosis involves numerous redundant and antagonist mechanisms to ensure the fidelity of chromosome segregation. Hence, to understand the roles that different motors play in spindle morphogenesis, it is necessary to develop in vitro models that mimic the microtubule organization found in the bipolar mitotic spindle, without interference from other force generating mechanisms. We are approaching this problem by creating artificial mitotic spindles in vitro that recapitulate the microtubule organization found in dividing cells. Microtubules are manipulated using AC electric fields generated by microfabricated chrome electrodes on quartz substrates. AC electric fields (3 x 10^6 V/m at 5 MHz) create both hydrodynamic flows and dielectrophoretic forces on the microtubules that cause the filaments to organize into aligned bundles on the electrodes. To sort the microtubules for polarity, neutravidin was patterned on the electrodes using deep-UV irradiation and segmented microtubules were prepared with biotinylated segments at the microtubule -ends. When accumulated by AC fields, microtubules with their -ends facing the electrodes bound, while filaments with their +ends facing the electrodes diffused away when the field was turned off. By placing opposed electrodes 15-25 microns apart, two oriented bundles of filaments are created, and the microtubules can be extended exclusively from their +ends using NEM-tubulin to create an overlap zone. When microscale beads functionalized with +end-directed kinesins interacted with these artificial spindles, they were transported away from the poles and displayed oscillatory motion around the midzone, similar to metaphase chromosomes. Establishing this complex organization of microtubules in vitro provides an important link between single-molecule investigations on individual motors and the complex dynamics driven by multiple motors in cells and cell extracts.
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1850/B312

Origins of Microtubule Bending in LLC-PK1 Epithelial Cells.

E. Tüzel[1], A. D. Bicek[2], A. Demtchouk[2], M. Uppalapati[3], W. O. Hancock[3], D. M. Kroll[4], D. J. Odde[2]; [1]Institute for Mathematics and Its Applications, University of Minnesota, Minneapolis, MN, [2]Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN, [3]Department of Bioengineering, Penn State University, University Park, PA, [4]Department of Physics, North Dakota State University, Fargo, ND

Microtubules are proposed to act mechanically as compressive struts that resist both acto-myosin forces and their own polymerization forces to mechanically stabilize cell shape. However, individual microtubules in living cells are often highly bent, and the origin of these deformations in living cells is still unknown. Here, we report a systematic analysis of MT deformation in the periphery of living LLC- PK1 cells, and assess the contribution of the different mechanisms, namely, polymerization, acto-myosin contractility and forces exerted by molecular motors resisted by passive cross-links. Our results strongly suggest that F-actin dynamics and polymerization of microtubules play a minor role, and that the dominant mechanism is anterograde transport via molecular motors. Interestingly, we found that in an in vitro kinesin-MT gliding assay, MTs buckle in a similar manner. To make quantitative comparisons, we measured curvature distributions of observed MTs and found that the in vivo and in vitro curvature distributions agree quantitatively. Motivated by these experiments, we have modeled the deformation of microtubules under the influence of molecular motor forces by coarse-grained simulations. In the simulations, microtubules are modeled as semi-flexible polymers with rigid bond constraints embedded in a Langevin solvent. Molecular motors exert forces on the microtubules, and walk along microtubule tracks according to their known force-velocity relations, bind and unbind stochastically. Preliminary results show that the distributions of curvatures for high density of molecular motors agree with the experimental data. Results support the idea that molecular motors are the dominant source of bending in living LLC-PK1 cells and argue against models in which MTs play a passive structural role, and instead favor a model in which active motor forces and cell shape control the spatial distribution of MTs.

1851/B313

Specificity of Spastin-Tubulin Interactions Required for Microtubule Severing.

S. White, S. Reisenweber, B. Lauring; Pathology and Cell Biology, Columbia University, New York, NY

Spastin, encoded by the most commonly mutated gene for Hereditary Spastic Paraplegia, is a microtubule severing enzyme. Although many other AAA+ ATPases have multiple target proteins, microtubules are the only known target of spastin. In exploring how spastin activity might be restricted to microtubules, we find that the tubulin C-terminal tail and ATP-dependent hexamerization are both important mediators

of the interaction between spastin and microtubules. The interaction between the tubulin C-terminus and the spastin pore does not require the clustered charged residues that are characteristic of all tubulin isoforms but is mediated by overall negative charge. When the C-terminal 22 amino acids of tubulin are replaced with a very similar acidic unstructured peptide (EB1), spastin binding is retained, whereas it is lost with a less similar sequence. Surprisingly, spastin does not bind EB1-C itself, suggesting spastin also interacts with a region outside the flexible tail of tubulin. The site of this second interaction maps to helices 11 and 12 of tubulin. Therefore, the specificity of spastin binding to microtubules is enhanced by multiple sites of contact. This detailed understanding of spastin-tubulin interactions may assist in determining the mechanism by which spastin severs microtubules.

1852/B314

Three-Dimensional Tracking of Growing Microtubule Ends by Two-Photon Microscopy.

T. Murata[1,2], S. Nonaka[1,2], T. Sano[3,4], S. Hasezawa[3,4], M. Hasebe[1,2]; [1]National Institute for Basic Biology, Okazaki, Japan, [2]School of Life Science, Graduate University for Advanced Studies (SOKENDAI), Hayama, Japan, [3]Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Japan, [4]Institute for Bioinformatics Research and Development (BIRD), Japan Science and Technology Agency (JST), Tokyo, Japan

Microtubules function in cellular organization and intracellular transport. They are often organized into a complex cytoskeletal array, such as a mitotic spindle, a midbody, and a phragmoplast, for proper functioning. Obsevation of individual microtubule behavior in the arrays facilitate to understand the mechanism of array organization, althouth it was difficult because of the complexity of the array with many microtubules growing and shrinking. Two-photon microscopy is useful for the observation with seeing structures embedded with excitation light-absorbing materials. We attempted three-dimensional tracking of growing microtubule ends by two-photon microscopy. We used tobacco suspension culture cells that stably expressed GFP-EB1, a marker of growing microtubule ends. At cytokinesis, the tobacco cells form a cell plate within a phragmplast, which contains two anitparallel microtubule arrays. Images of GFP-EB1 spots in a phragmoplast were taken in a three-dimensional stack, which is composed of more than 10 images at 0.3-0.5 micrometer intervals, using a piezo motor-driven objective lens. The stack was repeatedly recorded with 1.5 second intervals. After image processing and tracking of EB1 spots, we successfully determined sites of plus end appearance and paths of microtubule elongation. The method seems to be applicable to analysis of microtubule dynamics in other microtubule arrays.

1853/B315

Increased TBCB Expression Correlates with Low Microtubule Numbers in Reactive Microglia.

J. Villegas[2,3], M. Lopez-Fanarraga[1,3], G. Carranza[1,3], R. Castaño[1,3], J. Zabala[1,3]; [1]Biologia Molecular, Universidad de Cantabria, Santander, Spain, [2]Anatomia y Biologia Celular, Universidad de Cantabria, Santander, Spain, [3]IFIMAV, Santander, Spain

Recent lines of evidence suggest that tubulin cofactors could be important contributors to microtubule specialization and dynamics for they play key roles in tubulin dissociation, transitory tubulin storage and finally, in αβ-tubulin association. In particular, tubulin cofactor B (TBCB), which is not required in tubulin biogenesis, has recently been shown to participate during axonogenesis in the regulation of microtubule dynamics in the growth cone. We found that microglial cells of the amoeboid phenotype, typical of the developing nervous system, express abundant TBCB compared to most cell lineages in these primary CNS cultures. TBCB is upregulated upon activation in interferon treated BV-2 microglial cells, and in post-lesion reactive parenchymal microglia in vivo. Differentiation of amoeboid microglia to the ramified form is accompanied by a drop in TBCB expression and a more abundant microtubule network. Thus, our data suggest that TBCB controls microtubule numbers in these glial cells. We also found that upon TBCB gene silencing there is a significant rise in the microtubule content in amoeboid microglia with occasional microtubule changes, and that TBCB is not essential for microglial viability. These results define TBCB as a novel regulator of the number of microtubules in these glial cells, and provide an insight into the understanding of the complex mechanisms controlling microtubule reorganization during microglial transition between the amoeboid, ramified, and reactive phenotypes.

1854/B316

Curcumin Inhibits Cancer Cell Proliferation Apparently by Perturbing Microtubule Dynamics.

M. Banerjee, D. Panda; School of Biosciences and Bioengineering, Indian Institute of Technology Bombay, Mumbai, India

Curcumin, the active component of the spice turmeric, has emerged as a promising anticancer compound. Curcumin has been shown to bind to brain tubulin in vitro and inhibit microtubule polymerization both in vitro and in cultured cells. In this study, we examined the antiproliferative mechanism of action of curcumin and determined the probable binding site of curcumin on tubulin. Using EGFP-tubulin, we found that curcumin strongly dampened the dynamic instability of individual microtubules in living MCF-7 cells. It strongly reduced the rate and extent of shortening state while it modestly reduced the rate and extent of growing state. Curcumin strongly reduced the fraction of time microtubule spent in the growing state whereas increased the time microtubule spent in the pause state. At its half maximal proliferation inhibitory concentration (IC50), curcumin exerted strong suppressive effects on the dynamics of microtubules with minimal depolymerization of the interphase microtubules and appreciable depolymerization of spindle microtubules. However, at relatively high concentration (2 X IC50), curcumin significantly depolymerized both interphase and mitotic microtubules in MCF-7 cells. Curcumin increased nuclear accumulation of p53 in MCF-7 cells. Docking study performed using the Genetic Optimization for Ligand Docking (GOLD) program, revealed that curcumin shares an overlapping binding site with colchicine. The results indicated that curcumin inhibits cancer cell proliferation by perturbing microtubule dynamics. The work provides an insight into the binding site of curcumin on tubulin which may assist towards the development of potent curcumin analogues.

1855/B317

NIP2/Centrobin Is Critical for Mitotic Spindle Assembly and Its Activity Is Regulated By PLK1.

J. Park, J. Lee, H. Choi, K. Rhee; School of Biological Sciences, Seoul National University, Seoul, South Korea

NIP2/centrobin is a centrosomal protein which is critical for the MTOC activity in both interphase and mitotic cells. Here in vitro tubulin polymerization assay revealed that NIP2 nucleates and stabilizes microtubule. We observed NIP2 phosphorylation levels increased in mitotic cells. According to the results, we speculated that NIP2 activity on the spindle assembly might be regulated by phosphorylation. In search for a kinase for the phosphorylation, we found that NIP2 possesses conserved PLK1 phosphorylation sites, and the spindle abnormality of NIP2-suppresssed cells is reminiscent of one of phenotypes seen in PLK1-depleted cells. Consequently, we tested whether PLK1 regulates NIP2 for spindle assembly. The results showed that PLK1 phosphorylated NIP2 in vivo as well as in vitro. PLK1-suppression caused reduction in the NIP2 phosphorylation at mitosis. Finally, PLK1 phosphorylation enhanced the activity of NIP2 on microtubule nucleation in vitro. Based on these results, we propose that NIP2 is directly involved in microtubule nucleation and PLK1 positively regulates NIP2 to organize proper mitotic spindles.

1856/B318

Microtubule Assembly by Different Inducers Are Dependent on the Nucleation Stage.

N. E. Thomas, X. L. Louis, S. Sengupta; Molecular Medicine and Cancer Research, Rajiv Gandhi Centre for Biotechnology, Trivandrum, India

Microtubules are essential cytoskeletal polymers in all eukaryotes that engineer the perfect segregation of chromosomes during cell division. Further more, they serve as tracks for directed intracellular transport and they may play a role in locomotion and morphogenesis. Microtubule assembly from the heterodimeric protein tubulin passes through a rate determining nucleation stage which involves oligomer formation. In cells, nucleation stage plays an important role in the formation of microtubules. The polymeric stage and the dimeric stage are both well documented at the structural and functional level in living cells. But microtubule nucleation remains poorly understood. In this study, we have investigated the nucleation stage of microtubule assembly using different inducers. By spectrophotometry and electron microscopy, we found that the addition of different inducers like DMSO, Taxol, Epothilone and MAP2 at the end of the nucleation phase caused the induction of microtubule polymerization to the same extent, whereas the rate and extent of polymerization differed when the inducers were added initially along with tubulin. The oligomers formed after the nucleation stage, were purified by gel filtration chromatography by stopping the nucleation stage with a sulphydryl cross linker, EBI. The oligomeric species was confirmed by HPLC and non denaturing PAGE. The binding parameters of the anticancer drugs Taxol and Epothilone to the oligomers were measured by labeling the oligomers with an environment sensitive fluorescent probe, ANS. It was found that both Taxol and Epothilone bound to oligomeric tubulin with similar affinities which were much less than their affinities in the polymeric stage. This study proved that the polymerization ability of these inducers is highly dependent on the nucleation stage of microtubule assembly.

1857/B319

Centrosome-Independent Microtubule Generation within the Spindle Requires the 8-Subunit Augmin Complex and γ-Turc.

G. Goshima[1], S. Petry[2], R. D. Vale[2], R. Uehara[1]; [1]Institute for Advanced Research, Nagoya University, Nagoya, Japan, [2]Dept of Cellular and Molecular Pharmacology, UCSF, San Francisco, CA

The centrosome is the main site of microtubule nucleation in the animal mitotic spindle. gamma-tubulin, the major microtubule nucleator in cells, is enriched at centrosomes in mitosis. However, functional spindles can be formed without centrosomes in Drosophila, suggesting that a critical function for gamma-tubulin might reside elsewhere. Through a genome-wide RNAi screen and extensive follow-up experiments using Drosophila S2 cells, we identified eight genes (Dgt2-9) required for localizing gamma-tubulin to spindle microtubules. We show that the Dgt proteins interact, forming a stable complex. We find that spindle microtubule generation is substantially reduced after knockdown of each Dgt protein by RNAi. Thus, the Dgt complex that we name "augmin" functions to increase microtubule number. Reduced spindle microtubule generation after augmin RNAi, particularly in the absence of functional centrosomes, has dramatic consequences on mitotic spindle formation and function, leading to reduced kinetochore fiber formation, chromosome misalignment, and spindle bipolarity defects. We also identified human Dgt6 that is required for spindle microtubule generation in HeLa cells. Our results suggest that an important mitotic function for gamma-tubulin may lie within the spindle, where augmin and gamma-tubulin function cooperatively to amplify the number of microtubules.

1858/B320

Host Cell Sirna Screening Identifies a Critical Role for Host Microtubules in Toxoplasma Gondii Invasion.

K. R. Sweeney, I. Blader; Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, OK

Toxoplasma gondii is an obligate intracellular parasite that infects nucleated cells across a wide host range. *Toxoplasma* infections are typically asymptomatic, but infection can result in a disease called toxoplasmosis in fetuses and immune-compromised individuals, such as AIDS or transplant patients. During infection, parasites scavenge nutrients, modify host cell transcription and alter the host cell cytoskeleton. How parasites do this is largely unknown, and we hypothesized that parasite manipulation of host cell signaling pathways is likely to be involved. To identify host signaling pathways important for parasite growth, we screened siRNA libraries targeting 900 human kinases and phosphatases. From this screen, 27 host kinases and phosphatases were identified whose knockdown in expression resulted in a significant decrease in parasite growth. Signaling proteins identified in the screen included several kinases that interact with the microtubule cytoskeleton. Further experiments to determine the role these kinases play during parasite growth revealed there is a significant decrease in parasite invasion when these kinases are not expressed. Given that these signaling molecules interact with the microtubule cytoskeleton, we hypothesized that the host microtubule network was important for parasite invasion. To test this hypothesis, we measured parasite invasion using a synchronized *Toxoplasma* invasion assay. We found that host cell treatment with the microtubule destabilizing agents nocodazole and colchicine, which parasites are resistant to, significantly decreased parasite invasion of host cells. In support of this data, we found that microtubules were closely associated with the moving junction, which is where an invading parasite comes into contact with the host cell. Experiments are currently underway to define the mechanism by which host microtubules act to promote *Toxoplasma* invasion.

1859/B321

A Continuous Model for Microtubule Growth.

V. Rezania[1], P. Hinow[2], E. Carpenter[1], J. Tuszynski[1]; [1]Division of Experimental Oncology, University of Alberta, Edmonton, AB, Canada, [2]Institute for Mathematics and its Applications, University of Minnesota, Minneapolis, MN

In this study, we propose a new mathematical model to simulate dynamical behavior of microtubules in a pool of tubulin dimers by considering a time varying growth rate. Found in eukaryotic cells, tubulin polymerizes into microtubules which help provide structural stability and rigidity to cells, and play key roles in many physiological processes, including vesicle transport and mitotic chromosome separation. We show that the growth rate varies depending on the state of the GTP-cap, nucleation, catastrophic shortening, and rescue events. Our model, developed in terms of coupled partial differential equations with proper boundary conditions, respects the mass conservation law and captures all key features of the kinetic processes fundamental to the microtubule reorganization observed in living cells. The proposed model is a generalization of earlier attempts at describing the dynamic instability and it provides new insights into the underlying mechanisms of polymerization and depolymerization.

1860/B322

TIP150 Interacts and Targets MCAK to the Plus-End Microtubules.

K. Jiang[1,2], J. Wang[2], L. Wordeman[3], X. Yao[1]; [1]Cell Biology, Chinese University of Science & Technology, Hefei, China, [2]Physiology, Morehouse School of Medicine, Atlanta, GA, [3]Physiology, University of Washington, Seattle, WA

The microtubule cytoskeleton orchestrates cellular plasticity and dynamics underlying morphogenesis and cell diivision. Growing microtubule plus ends have emerged as dynamic regulatory sites in which specialized proteins, called plus-end-binding proteins (+TIPs), bind and govern the microtubule plus-end dynamics underlying cell division and migration. However, the molecular mechanisms underlying the plus-end regulation by TIPs at spindle microtubule and astral microtubule have remained elusive. Here we show that TIP150 is a novel plus-end-binding protein interacting with EB1. TIP150 binds directly to EB1 in vitro and colocalizes with EB1 at the astral microtubule plus-ends in vivo. Suppression of EB1 by siRNA eliminates the plus-end-binding of TIP150, indicating that EB1 specifies the plus-end localization of TIP150. Interestingly, TIP150 also interacts physically with MCAK, a microtubule depolymerase localizing to the plus-end of microtubule. Suppression of TIP150 abolishes the plus-end localization of MCAK, suggesting the importance of TIP150 in the plus-end targeting of MCAK. Importantly, Aurora B-mediated phosphorylation disrupts TIP150-MCAK association in vitro and at the spindle microtubule plus-ends in vivo. We reason that the TIP150 facilitates the EB1-dependent loading of MCAK onto microtubule plus ends and orchestrates the dynamics at the plus-end of microtubules.

1861/B323

In Vivo Role of MAP17, a New Subunit of the Spindle Regulatory Complex Augmin.

A. M. Meireles[1,3], K. Fisher[2,4], N. Colombié[1], J. Wakefield[2,4], H. Ohkura[1]; [1]Wellcome Trust Centre for Cell Biology, University of Edinburgh, Edinburgh, United Kingdom, [2]Department of Zoology, University of Oxford, Oxford, United Kingdom, [3]PDBEB, Center for Neurosciences and Cell Biology, University of Coimbra, Coimbra, Portugal, [4]Life Sciences Interface/Doctoral Training Centre, University of Oxford, Oxford, United Kingdom

Microtubule associated proteins (MAPs) are increasingly considered to be key regulators in the organisation of the spindle and microtubule (MT) network. Our MAPs-targeted RNAi screen identified several new MT regulators. One of them, MAP17, is a new component of Augmin, a recently discovered spindle regulatory complex in *Drosophila*. Experiments using *in vitro* translated proteins allowed us to define the region of MAP17 that directly interacts with one of the Augmin subunits. Similar to the depletion of other subunits of Augmin, depletion of MAP17 in S2 cells leads to a dramatic increase in the number of monopolar spindles and in number of longer spindles with reduced microtubule density. We have generated a deletion mutant of MAP17. Unexpectedly, the deletion mutant fly is viable, but female fertility is severely compromised. This suggests that MAP17 is not essential for somatic mitosis in flies but plays a crucial role in specialised cell divisions in oocytes and/or embryos.

1862/B324

Novel Role for Gamma-Actin in Microtubule Dynamics in Neuroblastoma Cells.

S. Po'uha[1], S. Honore[2], D. Braguer[2], M. Kavallaris[1]; [1]Pharmacoproteomics Program, Children's Cancer Institute Australia for Medical Research, Randwick, NSW, Australia, [2]FRE-CNRS 2737, CISMET, University of Marseille, Marseille, France

Tubulin-targeted agents disrupt microtubule dynamics, inducing mitotic arrest and cell death. These agents are highly effective in the treatment of cancer however, development of drug resistance can diminish their therapeutic effect. It is now established that alterations in the tubulin/microtubule system can perturb the action of tubulin-targeted agents. We recently reported novel mutations in, and altered expression of the cytoskeletal protein γ-actin in drug resistant leukemia cells (1). Moreover, γ-actin was found to be functionally involved in mediating response to tubulin-targeted agents and paclitaxel induced tubulin polymerization. Since microtubule stability can be modulated by the actin cytoskeleton, we hypothesized that γ-actin can modulate microtubule dynamics, altering cellular response to tubulin-targeted agents. Herein, we examined the effect of γ-actin on microtubule dynamics in neuroblastoma SH-EP cells expressing GFP-βI-tubulin. The plus end of individual microtubules in interphase cells were tracked over time using the Metamorph software. Interestingly, siRNA knockdown of γ-actin expression significantly suppressed microtubule dynamics by 17.4% compared to the control siRNA transfectants. Suppression of microtubule dynamics in the γ-actin knockdown cells was associated with a slow shortening rate, longer pause duration and the microtubules spending more time in attenuation and less time in growing events. To determine whether knockdown of γ-actin affects paclitaxel induced mitotic arrest, the siRNA transfected cells were treated with increasing concentrations of paclitaxel for 22 hours and stained with DAPI to visualize the chromosomes. In contrast to a dose-dependent increase in mitotic index of the control siRNA transfectants, knockdown of γ-actin moderately inhibits paclitaxel induced mitotic arrest. This is the first demonstration that γ-actin can regulate microtubule dynamics and directly implicates γ-actin in the ability of tubulin-targeted agents to induce mitotic arrest. 1. Verrills NM, Po'uha ST, Liu ML, et al. Alterations in gamma-actin and tubulin-targeted drug resistance in childhood leukemia. J Natl Cancer Inst 2006;98:1363-74.

1863/B325

Building a Spindle of the Correct Length in Human Cells Requires the Interaction between TPX2 and Aurora A.

A. W. Bird, A. A. Hyman; Max Planck Institute - CBG, Dresden, Germany

To assemble mitotic spindles, cells organize microtubules nucleated from a variety of sources, including chromosomes and centrosomes. We know little about how the regulation of microtubule nucleation contributes to spindle bipolarity and spindle size. The Aurora A kinase activator TPX2 is required for microtubule nucleation from chromosomes as well as for spindle bipolarity. Aurora A kinase itself is required for many different mitotic processes, reflecting the complexity of kinase regulation through specific activators. We have used bacterial artificial chromosome (BAC) -based recombineering to introduce point-mutants that block the interaction between TPX2 and Aurora A into human cells to examine the role of TPX2-mediated Aurora A activation in spindle assembly. These TPX2 mutants have very short spindles, but in contrast to cells depleted of TPX2, they are still bipolar and segregate chromosomes. While spindle poles normally separate after nuclear envelope breakdown (NEBD) until they reach a characteristic distance apart, we found that in TPX2 mutants spindle poles immediately collapse towards each other after NEBD, and are unable to fully separate to form a spindle of appropriate length. Thus it appears determinants of spindle length are operating immediately after NEBD. Consistent with this we found that hyper-stabilized microtubule populations in prometaphase are less stable and disorganized when Aurora A activation by TPX2 is abolished. Furthermore, examination of microtubule nucleation during spindle assembly in TPX2 mutants shows that microtubules fail to nucleate from chromosomes. Thus chromosome nucleation is not essential for bipolarity during human cell mitosis when centrosomes are present. Rather chromosome nucleation is involved in spindle pole separation and setting spindle length. We propose that the amount of Aurora A and its activation on the spindle determines chromatin-based microtubule nucleation and spindle length, and this is regulated by TPX2.

1864/B326

Evidence for an Upper Limit to Mitotic Spindle Length.

M. Wuehr[1], Y. Chen[2], S. Dumont[1], A. Groen[1], D. Needleman[1], A. Salic[2], T. Mitchison[1]; [1]Systems Biology, Harvard Medical School, Boston, MA, [2]Cell Biology, Harvard Medical School, Boston, MA

Size specification of macromolecular assemblies in the cytoplasm is poorly understood. In principle, assemblies could scale with cell size, or use intrinsic mechanisms to achieve fixed, but regulated, sizes. For the mitotic spindle, scaling with cell size is expected, since the function of this assembly is to physically move sister chromatids into the center of nascent daughter cells. Anecdotally, spindle length does scale with cell length, but it is not clear if this scaling mechanism could operate at very large cell lengths. Eggs of Xenopus laevis are among the largest cells known that cleave completely during cell division. Cell length in this organism changes by two orders of magnitude (~1200 µm to ~12 µm) while it develops from a fertilized egg into a tadpole. We wondered if, and how, mitotic spindle length and morphology adapt to function at these different length scales. Here, we show that spindle length increases with cell length in small cells, but in very large cells spindle length approaches an upper limit of ~60 µm. To transport the DNA into the center of the daughter cells, the relatively small spindle length is compensated by an enormous anaphase B-like movement. Further evidence for an upper limit to spindle length comes from an embryonic extract system that recapitulates mitotic spindle assembly in a test tube. We conclude that early mitotic spindle length in Xenopus laevis is uncoupled from cell length, reaching an upper bound determined by mechanisms that are intrinsic to the spindle.

1865/B327

The Heterotrimeric G Protein, Gsα, Modulates Microtubule Dynamics by Stimulating Tubulin GTPase Activity.

R. Dave'[1], M. Lopus[3], R. Luduena[2], L. Wilson[3], M. M. Rasenick[1]; [1]Physiology, University of Illinois-Chicago College of Medicine, Chicago, IL, [2]Biochemistry, U. Texas Health Science Center, San Antonio, TX, [3]Cell Biology, University of California, Santa Barbara, CA

Clinically, microtubules are involved not only in cancer but may play a role in neurologic and psychiatric disorders, including depression. Indeed, antidepressant response involves formation of new synapses, and this may depend upon both Gsα and microtubules. Gα-tubulin interactions are required for neurite outgrowth. Gsα binds tubulin (100 nM affinity) in regions of active neurite formation such as dendritic spines and post-synaptic densities. Moreover, a Gα protein dominant negative for tubulin interactions prevents neurite outgrowth in primary hippocampal neurons. However, a functional understanding of Gsα-tubulin interactions at a molecular and structural level has remained unclear. Here, we show that only active Gsα binds tubulin. Additionally, Gsα differentially interacts with brain tubulin isotypes. Gsα decreases microtubule stability by stimulating tubulin GTPase activity and sequestering tubulin-GDP. Gsα binds to both soluble tubulin and ovine brain microtubules and Gsα destabilizes microtubules in a manner somewhat akin to colchicine (despite very different putative binding sites on tubulin). Evidence is presented from biochemical as well as imaging approaches. Regions of Gsα involved in functional interactions were investigated. Previous modeling and peptide array studies proposed a role for the α3 helix, α3-β5 loop and β5 sheet of Gsα in binding to tubulin. Mutational analysis indicates a role for the α3-β5 loop in modulating tubulin GTPase activity and microtubule stability. Interestingly, this region is also involved in Gsα's interaction with adenylyl cyclase and Gβγ. Taken together, these data are consistent with a model suggesting that, upon activation by neurotransmitter, Gsα internalizes on lipid-raft-derived vesicles. These vesicles deliver a high concentration of active Gsα to tubulin at the end of microtubules. There, active Gsα increases microtubule dynamics by stimulating tubulin GTPase on the microtubule and sequestering released tubulin-GDP. The interaction with tubulin is limited by the autohydrolysis of GTP on Gsα. In this manner, Gsα increases microtubule dynamics, and promotes both neurite outgrowth and the formation of new neurites. Support: NIMH, NIDA

1866/B328

A Peptide Corresponding to the Interface between Gsα and Tubulin Activates the Intrinsic Tubulin GTPase and Increases Dynamic Instability of Microtubules.

w. Saengsawang[1], M. Lopus[2], L. Wilson[2], M. M. Rasenick[1]; [1]Physiology and Biophysics, University of Illinois at Chicago, Chicago, IL, [2]Molecular, Cellular, and Developmental Biology and The Neuroscience Research Institute, University of California, Santa Barbara, Santa Barbara, CA

Gsα, the stimulatory G protein, interacts with tubulin with high affinity (Kd~115nM). Gsα has been shown to increase tubulin GTPase activity and destabilize microtubules. In addition, expression of mutagenically activated Gsα stimulates neurite-like outgrowth in PC12 cells. N-terminal, α2-β4 and α3-β5 regions on Gsα have been suggested as potential regions that interact with tubulin. The purpose of this study is to determine the effect of peptides from these regions on tubulin and microtubule dynamics. Surface Plasmon Resonance was used to determine the affinity of the peptides to tubulin. Single turnover GTPase activity assay was used to determine the effect of peptide on tubulin GTPase. The effect of peptides on microtubule dynamics was studied by tracking the microtubule growth from sea urchin axoneme seeds. In addition, the effect of Gsα-derived peptide on microtubule was determined with electron microscopy using negative staining for microtubule. We found that a Gsα-derived peptide from α3-β5 region binds specifically to tubulin and mimics Gsα stimulating tubulin GTPase. This peptide also increases microtubule dynamics; specifically by increasing catastrophe frequency. Furthermore, the effect of peptide on destabilizing microtubules was observed by electron microscopy revealing decrease in amount of microtubule polymerized in the present of Gsα derived-peptide. Peptide from transducin (Gtα) did not bind to tubulin and has no effect on microtubules. Replacing specific amino acid residues from Gtα to Gsα peptide leads to decrease in the effect of this peptide on tubulin GTPase activity, suggested that specific amino acid residues crucial for Gsα peptide (as well as Gsα) activation of tubulin GTPase. These results indicated that the small molecule from specific region of Gsα mimics the effect of Gsα on tubulin GTPase. As a result, it increases microtubule dynamics as well as destabilizes microtubule. This study provides insight into the effect of Gsα on microtubule dynamics and may help us to understand how hormones or neurotransmitters can alter cytoskeleton processes including cell migration or cell morphology. This peptide can also provide potential targets for future drug development.

1867/B329

The Role of Murine CENP-F Interaction with Hook2 in Centrosomal Microtubule Dynamics.

K. Moynihan, R. Pooley, D. Bader; Cell and Developmental Biology, Vanderbilt University, Nashville, TN

The centrosome is the microtubule organizing center (MTOC) of the cell and plays a primary role in cell division and motility. The dynamic nature of the MTOC is paramount in coordinating the mitotic spindle. Earlier in the cell cycle, the centrosome must duplicate and migrate and then repolymerize microtubules (MTs) from each new pole of the cell during prophase. Centrosome processes such as duplication, migration, maturation, and MT nucleation and anchor require specific proteins to ensure proper cycle behavior. The linker protein Hook2 has been shown to localize to the centrosome, bind centrosomal protein centriolin, and function specifically in MT organization and growth. Disruption of Hook2 expression leads to aberrant localization of centrosomal proteins ninein and PCM-1 and interference with MT radial organization and repolymerization. This phenotype suggests an important role in the centrosome regulation of MTs. Murine CENP-F (formerly called LEK1) has also been shown to participate in MT regulation with NudE and the LIS1 pathway. Additionally, human CENP-F promotes proper kinetochore attachment to the mitotic spindle, has a MT binding domain, and is used as a marker of proliferative cells. Knockdown of mCENP-F results in MT network collapse around the nucleus and disrupted protein expression attenuates MT repolymerization after nocodazole challenge. We have discovered an important interaction between these two regulators of the MT network; the N-terminus of mCENP-F binds the coiled coil region of Hook2. Thus far, a yeast two-hybrid (Y2H) screen, biochemical analyses, and immunofluorescence co-localization studies provide ample evidence supporting this physical interaction. Furthermore, Y2H analysis has identified the specific binding domain of each protein. Uncoupling this interaction in cell culture dramatically slows MT repolymerization after nocodazole treatment. Given the importance of MT fluctuation in the mitotic spindle, this interference with MT dynamics potentially extends to an impediment to cell division. The interaction between these two proteins is a possible physical link from the MT network to the centrosome and this may underlay multiple processes regulated by the MT network.

1868/B330

Growth Factor Signaling Promotes the Rapid Regrowth of Microtubules and the Accumulation of γ-Tubulin at the Centrosome during Interphase.

D. Colello, S. LaFlamme; Center for Cell Biology and Cancer Research, Albany Medical College, Albany, NY

Microtubule assembly from the centrosome regulates the localization of organelles, promotes vesicular transport and directs cell migration during interphase. To examine the contribution of growth factor signaling in microtubule assembly, a regrowth assay was used in which microtubules were depolymerized with nocodazole, and then allowed to reassemble following the removal of nocodazole. For these studies, serum-starved cells were adhered to an extracellular matrix protein in either serum-free medium or the growth-promoting medium, CCM1. The results indicate that rapid initiation of microtubule assembly from the centrosome requires growth factor signaling. Similar results were obtained with both Chinese hamster ovary (CHO) cells and primary human fibroblasts (HFSF), indicating that the effects are not cell type-specific. Interestingly, adding androgen, a component of CCM1, to serum-free medium was sufficient to promote rapid microtubule regrowth. Importantly, the effects of androgen were observed after 5 minutes, indicating that the ability of androgen to promote microtubule regrowth was likely to be independent of its ability to regulate transcription. Furthermore, androgen receptor siRNA inhibited rapid microtubule regrowth in response to CCM1, confirming a role for androgen receptor signaling. In addition, androgen promoted the accumulation of γ-tubulin at the centrosome suggesting that these processes may be mechanistically linked. Current studies are focused on determining whether androgen enhances microtubule nucleation and whether it does so by altering posttranslational modifications of γ-tubulin to promote its recruitment to the centrosome.

1869/B331

Reorganization of Microtubular Network during Endocytosis.

M. Kharchenko, M. Zlobina, E. Kornilova; Membrane Dynamics Lab, Institute of Cytology Russian Academy of Science, St. Petersburg, Russian Federation

Microtubular network in the cell is considered to be unchangeable in interphase. Thus microtubules (MTs) are supposed to serve as passive rails for endosomal transport by means of motor proteins. However, our results don't correspond with this hypothesis. We observed

endocytosis of Epidermal Growth Factor Receptor (EGFR) in certain lines of fibroblasts and epithelial cultural cells. These cells possessed radial MT cytoskeleton in interphase. By means of indirect double immunofluorescence and subcellular fractionation we revealed that stimulation of EGFR endocytosis resulted in reorganization of MT network. MT system remained radial when EGFR was located in early endosomes. EGFR translocation from early endosomes to the late ones and to lysosomes was accompanied by fragmentation and (or) depolimerization of MTs. The degree of this reorganization correlates with the rate of EGFR translocation. Later on we observed accumulation of EGFR degradation products in the incubation medium and reestablishment of radial MT network. These changes didn't depend on actin cytoskeleton morphology. Disruption of actin cytoskeleton by Cytochalasin D fails to abolish MTs reorganization. According to these data we suppose MT to facilitate endosome maturation and interaction with lysosomes.

1870/B332

A Novel Role of Gamma-Tubulin Ring Complexes in Microtubule Plus-End Dynamics in Drosophila Cells.

C. Verollet[1], A. Bouissou[1], A. Sousa[2], P. Sampaio[2], M. Wright[1], A. Merdes[1], C. Sunkel[2], B. Raynaud-Messina[1]; [1]UMR2587, CNRS, Toulouse, France, [2]IBMC-Instituto de Biologia Molecular e Celular, Universidade do Porto, Porto, Portugal

Nucleation of microtubules is supported by gamma-tubulin, a protein that assembles in two major complexes: the gamma-Tubulin Small Complex (gamma-TuSC) and the gamma-Tubulin Ring Complex (gamma-TuRC). In Drosophila cells, proteins of the gamma-TuSC are essential for viability and assembly of a functional mitotic spindle while gamma-TuRC-specific components are non-essential but required for efficient mitotic progression and organization of cell type-specific microtubule networks (Verollet et al., 2006, J. Cell Biol. 172:517-528). However, little is known about the specific function of these different gamma-tubulin complexes during interphase. Unexpectedly, we find that RNAi against gamma-TuRC proteins reduces the stability of microtubule plus-ends, independently of an effect on nucleation. Consistent with these data, the depletion of any component of the gamma-TuRC induces an accumulation of the plus-end tracking protein EB1 on microtubules. Moreover, a fraction of the gamma-TuRC localizes along the surface of cytoplasmic microtubules. We show that gamma-tubulin complexes are involved in controlling microtubule dynamics, in addition to their well-characterized role in nucleation.

1871/B333

Microtubule Cytoskeleton Remodeling by Acentriolar Mtocs during Mitotic Spindle Assembly and Disassembly in Animal Cells.

S. Moutinho Pereira[1], A. Debec[2], H. Maiato[1,3]; [1]Instituto de Biologia Celular e Molecular, Porto, Portugal, [2]CNRS- Equipe Polarité et Morphogenèse, Institut Jacques Monod Universités, Paris, France, [3]Lab. Cell and Molecular Biology, Faculdade de Medicina, Universidade do Porto, Porto, Portugal

Cytoskeleton microtubules undergo a reversible metamorphosis as cells enter and exit mitosis to build a transient mitotic spindle required for chromosome segregation. Centrosomes play a dominant but dispensable role in microtubule organization throughout the animal cell cycle, supporting the existence of concurrent mechanisms that remain unclear. Here we show that acentriolar microtubule-organizing centers (aMTOCs) containing γ-tubulin ring complexes play an active role during spindle morphogenesis in Drosophila somatic cells. Some aMTOCs are recruited to acentriolar spindle poles by an Ncd/dynein-dependent microtubule clustering mechanism forming rudimentary aster-like structures. Studies of taxol-induced microtubule aster formation and spindle disassembly in normal or colchicine-treated cells suggest that aMTOCs can be recycled from spindle-associated γ-tubulin. Remarkably, after anaphase onset several microtubules are nucleated de novo from aMTOCs as a direct consequence of CDK1 downregulation. Our data reveal a new form of cell-cycle regulated MTOCs important for centrioleindependent microtubule cytoskeleton remodeling during mitotic spindle assembly and disassembly in animals

1872/B334

Can the FtsZ Constriction Force be Achieved without GTP Hydrolysis?

M. Osawa, D. E. Anderson, H. P. Erickson; Cell Biology, Duke University Medical Center, Durham, NC

The tubulin homolog FtsZ forms the cytokinetic Z ring that divides bacteria. We have recently reconstituted Z rings in liposomes, using a membrane-targeted FtsZ, and demonstrated that they can generate a constriction force without any other proteins (Science 320:792-4, 2008). One mechanism suggested for this force generation is the conformational change of FtsZ protofilaments. FtsZ protofilaments can adopt a straight conformation equivalent to those in a microtubule wall, or a curved conformation with a diameter of 23 nm equivalent to the tubulin rings that peel from the microtubule during depolymerization. GTP and GDP favor the straight and curved conformations, respectively. Thus GTP hydrolysis could induce a transition from the straight to curved conformation, and this could generate a constriction force if the protofilaments are tethered properly to the lipid membrane. To explore this mechanism we tested the analog GMPCPP, which is hydrolyzed by FtsZ 50 times slower than GTP (0.08 GMPCPP per min per FtsZ, vs. 4 per min for GTP). As a further test we explored the mutant FtsZ-D212G, which has been reported to be highly deficient in GTP hydrolysis (but still functional for cell division). We purified D212G protein by a multi-step protocol to eliminate contaminating GTPase's, and determined a hydrolysis rate of 0.02 per min per FtsZ. Both wtFtsZ + GMPCPP, and D212G + GTP assembled Z rings in liposomes, and the Z rings generated constrictions. These two experiments suggest that the constriction force may not require GTP hydrolysis. However, we have so far observed the constrictions only about ten minutes after mixing FtsZ, GTP and lipids. This would be time enough for one full round of hydrolysis by FtsZ + GMPCPP. For D212G, however, a maximum of one in five subunits would have hydrolyzed a GTP, so this is likely constriction without GTP hydrolysis.

Centrosomes II (1873 – 1890)

1873/B335

Functional Characterisation of Three Novel Human NIMA-related Kinases: Nek3, Nek5 and Nek11.

N. K. Sahota, A. M. Fry; Biochemistry Department, University of Leicester, Leicester, United Kingdom

Approximately 2% of the human kinome is composed of a poorly characterised family of eleven serine/threonine kinases related to the NIMA kinase of *Aspergillus nidulans*. NIMA is required for mitotic entry and its overexpression drives cells into a premature mitosis from any point in the cell cycle. The human kinases, named Nek1 to Nek11, share a common protein structure with an N-terminal catalytic domain and a C-terminal regulatory domain that is highly variable in sequence and length. Of the eleven human Neks, Nek2 is most closely related to NIMA and is the most extensively studied. Like NIMA, it also contributes to the G_2/M transition through regulating centrosome separation and bipolar spindle assembly. Similarly, there is evidence that Nek6, Nek7 and Nek9 play roles in mitotic progression and spindle formation. In contrast, Nek1 and Nek8 are implicated in ciliogenesis as their mutation can lead to polycystic kidney disease, a typical ciliopathy phenotype. The common underlying theme between spindle formation and ciliogenesis is the role of microtubules and microtubule organizing centres and several Neks, both in humans and lower eukaryotes, localize to centrosomes. Hence, the current hypothesis is that all Neks may contribute to MT organisation in dividing and/or differentiated cells. However, the remaining human Neks are much less well studied and have no assigned functions. The objective of this research is therefore to provide a first comprehensive analysis of three of these kinases, Nek3, Nek5 and Nek11. For this purpose, we aim to study their expression, localisation, activity and function in dividing and ciliated cells. Results obtained so far indicate that all three kinases are expressed in U2OS, HEK293 and RPE1 cells. We have generated constructs for expression of active and inactive kinases in cells and optimized kinase assays for Nek3 and Nek11. We have also now raised specific antibodies against Nek5 and Nek11 that will allow us to examine the localization and activity of the endogenous proteins. The generation of these reagents will now enable us to examine the properties, regulation and function of these three novel members of the Nek kinase family in cell cycle progression and ciliogenesis.

1874/B336

Regulation of Maskin by Aurora a during the Spindle Assembly.

I. Peset Martin, T. Sardon, I. Vernos; Cell Biology and Development, CRG, Barcelona, Spain

Maskin belongs to the tranforming acidic coil-coiled (TACC) family of proteins. They have been emerged as important players for spindle assembly. Studies performed in Xenopus egg extract, including our work, have shown that Maskin promotes microtubule elongation from the centrosome. Maskin recruits the microtubule stabilizing factor XMAP215 to the centrosome and enhances its activity. We have now examined whether Maskin has also a role in the chromatin-dependent microtubule aster formation pathway. We found that in the absence of Maskin this pathway is still active although there is a delay suggesting that Maskin is not essential but participates in its kinetics. We have previously shown that Maskin is phosphorylated by Aurora A and that this phosphorylation is important for its localization and function at the centrosome. We now found that Aurora A phosphorylation is not required for Maskin function in the chromatin dependent pathway. To understand better the role of phosphorylation In Maskin regulation, we have performed experiments interfering with Aurora A activity in egg extract. Aurora A depletion impaired Maskin localization to the centrosome and this was rescued by addition of wt but not KD Aurora A. We have also developed a new tool based on the activation of Aurora A by TPX2 that helped us to clearly define the requirements for localization and activation of the kinase. Addition of the N-terminus of TPX2 (GST-NT) to the egg extract results in the efficient activation of endogenous Aurora A kinase and prevents its localization to the centrosome and spindle microtubules. Using an anti-Maskin phospho-specific antibody directed against the conserved Ser626 we found that phosphorylated Maskin increases three folds in egg extract containing GST-NT. Interestingly, this does not result in a more efficient recruitment of Maskin to the centrosome and spindle. Our results suggest that the recruitment of Maskin to the centrosome not only requires its phosphorylation by Aurora A but also a direct protein-protein interaction with the kinase or with other proteins at the centrosome.

1875/B337

The Novel Cartwheel Protein Poc1 Acts in Centriole Assembly and Organization.

C. Pearson, T. H. Giddings, M. Winey; MCDB, CU at Boulder, Boulder, CO

The early structural intermediates leading to basal body and centriole assembly are analogous and well described morphologically. However, little is known about their molecular constitution and dynamic assembly. We utilized a Tetrahymena proteomics screen to identify novel proteins in cartwheels, an early assembly intermediate. Nineteen proteins were localized to distinct ultrastructural basal body domains and four components were found at cartwheels. Using high resolution fluorescence and electron microscopy, we monitored the assembly dynamics of proteins at discrete basal body structural domains. Protein incorporation at basal bodies ranges from complete dynamic exchange through the cell cycle to conservative incorporation only during new assembly. Two cartwheel components, Poc1 and the Sas-6 family member, TtSas6a, exhibit bimodal assembly in which the majority of each protein incorporates only during new basal body assembly while a smaller fraction exchanges with the cytoplasmic pool through the cell cycle. Poc1 is a novel cartwheel component that assembles at basal bodies coincident with new basal body assembly in a manner that is dependent upon its WD40 repeat. Tetrahymena Poc1 is required for normal basal body assembly and organization and Poc1 loss results in basal body structural defects. Two Poc1-like proteins are evident in humans, HsPoc1a and HsPoc1b. As expected for cartwheel proteins, HsPoc1 localizes to the proximal end of centrioles in human tissue culture cells. Depletion of HsPoc1 decreases centrosome reduplication in S-phase arrested cells. Furthermore, depletion of HsPoc1b, but not HsPoc1a, inhibits primary cilia formation.

1876/B338

Investigating the Functions of the Centrosomal Proteins Ana1, 2 and 3 in Drosophila.

N. R. Stevens, J. Dobbelaere, J. Raff; Gurdon Institute, Cambridge, United Kingdom

Centrioles are complex tubular structures, which are arranged as an orthogonal pair at the core of the centrosome, and are required for centrosome formation. It is centriole replication that drives the duplication of the centrosome prior to mitosis but despite the fundamental nature of this process and the fact that it frequently malfunctions during tumourigenesis it remains poorly understood. Only three proteins, DSas-4, DSas-6 and Sak, are known to be required for centriole replication in flies. However recently three new candidates were proposed on the basis of a genome wide RNAi screen in *Drosophila* tissue culture cells for genes involved in spindle assembly (Goshima et al., 2007). RNAi of these previously uncharacterised genes resulted in a high level of anastral spindles and they were therefore named *Ana1*, *2* and *3*.

That these genes could encode centriole replication factors is supported by a similar screen carried out in our lab where it was found that RNAi of *Ana1*, *2* and *3* leads to a reduced number of centrioles in tissue culture cells (J. Dobbelaere, unpublished). We are taking several approaches to characterize the three Anas in flies and investigate their possible roles in centriole replication. Firstly we are generating flies mutant for these genes to study their function *In Vivo*. We have raised antibodies and generated GFP fusions, which have allowed us to determine that the three Anas localize to distinct regions of the centriole. In addition we have produced transgenic flies that allow us to test the ability of the three proteins to drive centriole over-duplication when over-expressed in various tissues. When over-expressed in unfertilised eggs Sak, DSas-6 and DSas-4 can all drive *de novo* centriole formation. We have found that only Ana2 is capable of inducing this phenomenon. By comparing the results of these experiments on the Anas with existing data for Sak, DSas-6 and DSas-4 we should be able to outline a pathway for centriole assembly in *Drosophila*. So far it appears that the mechanism of centriole replication has been highly conserved across evolution and it is therefore to be hoped that what is learned from studying the Anas will similarly be applicable to humans.

1877/B339

De Novo Centriole Formation in Xenopus Egg Extract.

E. Hatch[1], T. Stearns[1,2]; [1]Department of Biology, Stanford University, Stanford, CA, [2]Department of Genetics, Stanford School of Medicine, Stanford, CA

Centrosomes in dividing animal cells comprise two centrioles surrounded by the microtubule-nucleating pericentriolar material. Centriole duplication is temporally limited to once per cell cycle and spatially limited to existing centrioles. The temporal control is mediated in part by disengagement of the mother and daughter centrioles at the metaphase-anaphase transition, which appears to be required for timely formation of new centrioles in the subsequent cell cycle. It is not known how disengagement in mitosis promotes new centriole formation, or how new centriole formation is spatially controlled. The kinase Plk4 is required for centriole formation and might be important for regulating centriole number: Plk4 overexpression causes centriole overduplication and can lead to de novo formation of centrioles, bypassing both temporal and spatial controls. How Plk4 effects centriole formation is unclear. We developed a system for studying Plk4-based centriole formation in Xenopus egg extract. We find that overexpression of the Xenopus Plk4 ortholog Plx4 causes overduplication of sperm centrioles as well as de novo centriole formation in the absence of centrioles. De novo centriole formation requires Plx4 kinase activity, demonstrated using a kinase-dead mutant of Plx4. We are now exploiting this system to understand the role of Plk4 in centriole formation and how its function is coordinated with the other cell cycle events of the centriole duplication pathway.

1878/B340

New Centrosome Components Identified by Analysis of the Mammalian Ciliogenesis Transcriptome.

R. Hoh[1], T. Stearns[1,2]; [1]Dept. of Biology, Stanford University, Stanford, CA, [2]Dept. of Genetics, Stanford University Medical School, Stanford, CA

We sought to identify and characterize new structural and regulatory components of centrioles and cilia using ciliated epithelial cells as a model system. Multiciliated tracheal epithelial cells (MTECs) produce hundreds of centrioles and motile cilia during differentiation considering that typical cells have only a single non-motile cilium and a pair of centrioles, this differentiation must be accompanied by transcriptional upregulation of centriole and cilium components. To characterize the transcriptional profile of ciliogenesis, we have used a ciliated cell-specific marker, FoxJ1-GFP, to sort ciliating from non-ciliating cells in mouse tracheal epithelial cultures differentiated in vitro. Samples were taken at two timepoints corresponding to distinct stages in differentiation: 4 days after the establishment of air-liquid interface (ALI), when most FoxJ1-GFP-positive cells are undergoing centriole duplication, and 12 days post-ALI, when the majority of FoxJ1-GFP-positive cells are fully ciliated. Transcriptional data from microarray analysis were subtracted against FoxJ1-negative populations to identify approximately 1500 genes that are differentially regulated specifically in ciliating cells. Comparison of our dataset with existing ciliary, centrosome and basal body proteome data shows that many of the most highly upregulated genes identified in our dataset are known centrosome and cilia components. Furthermore, regulators of centriole duplication such as Plk4, CenpJ (the mouse ortholog of Sas-4) and Sas-6, were among the genes whose expression was highest at day 4 and decreased the most at day 12. Using this profile as a criterion for choosing previously uncharacterized proteins to study, we have identified new components of the centrosome. These localize to distinct substructures of the centrosome, and we are now using several approaches to determine their function in the centrosome in general, and specifically during centriole formation in multiciliated cells.

1879/B341

How Is Mitotic Spindle Length Set in C. Elegans?

G. Greenan, A. A. Hyman; Max Planck Institute - CBG, Dresden, Germany

The mitotic spindle is a highly organised microtubule array that separates the sister chromatids during mitosis. Failure to assemble a functional spindle of the correct size can lead to chromosome segregation defects. Previous studies have found that chromatin, microtubule motors, and proteins that influence microtubule dynamics can affect the length of a spindle. However it remains unclear which pathways a cell actively uses to determine its spindle length. We used the highly invariant embryonic divisions of the nematode, Caenorhabditis elegans to study this problem. In C. elegans embryos the centrosomes are the major microtubule organising centres during mitosis and they form the poles of the mitotic spindle. Our analysis of the early mitotic divisions revealed a linear relationship between centrosome size and spindle length. Using RNAi against PCM components to generate smaller centrosomes, we were able to show that centrosome size set spindle length. In order to determine which centrosome proteins were responsible, we used RNAi to target specific centrosome protein complexes, and looked at the effect on spindle length. Interestingly, the TPXL-1/Aurora A kinase pathway had the most pronounced effect on spindle length. As TPXL-1 was gradually depleted, spindles got progressively shorter, and the amount of TPXL-1 on the centrosome correlated with this decrease in spindle length. Furthermore, when we looked at spindles through development, the amount of TPXL-1 on the centrosomes decreased as spindles got shorter. We propose a model where the amount of TPXL-1/Aurora A kinase on the centrosome is determined by centrosome size, and this regulates microtubule dynamics and ultimately spindle length.

1880/B342

APC/C-Cdh1 Regulates Centrosomin Destruction.

R. T. Jones, T. Megraw; Pharmacology, University of Texas Southwestern Medical Center Dallas, Dallas, TX

Assembled prior to mitosis and disassembled in G1, the mitotic centrosome is a dynamic structure that directs assembly of spindle microtubules. The pericentriolar matrix (PCM), assembled around a centriole core, consists of a milieu of proteins, and its assembly at mitosis (centrosome maturation) is critical to centrosome function in mitosis. In Drosophila, Centrosomin (Cnn) is required for centrosome maturation and the recruitment of all PCM components examined so far. The regulation of Cnn localization at mitosis onset is therefore pivotal to centrosome maturation. In a reversal of this process, PCM components are delocalized from the centrosome upon mitotic exit and as cell cycle progression into G1 occurs. Controlled degradation is a potent mechanism for regulating the cell cycle. At mitosis, the anaphase promoting complex/ cyclosome (APC/C) achieves this activity, a process conserved in all eukaryotes. Dictating substrate specificity and timing, the adapter proteins Cdc20 and Cdh1 regulate APC/C, with Cdc20 governing early mitotic events and Cdh1 targeting late substrates. Cdh1 localizes to the centrosome throughout mitosis affording it access to PCM components. In this study, we demonstrate that Cnn is ubiquitylated by APC/C-Cdh1 at a "KEN box" motif, a Cdh1 degron sequence, located at amino acid position 354 of Cnn. Expression of the KEN box-mutated, "degron dead", form of Cnn resulted in multiple Cnn puncta that contain other centrosome proteins and that function as small microtubule organization centers during mitosis. Despite this disruption of centrosome structure, the cell cycle was unaffected and bipolar mitotic spindles formed in most cases. Remarkably, this centrosome phenotype was phenocopied upon RNAi knockdown of Cdh1. Together, these data indicate that regulation of Cnn destruction by APC/C-Cdh1 is essential to maintain or organize centrosome structure.

1881/B343

AKAP450 Maintains Centrosome Cohesion via Its Interaction with CDK5RAP2, a Protein Mutated in Primary Microcephaly.

A. Barr[1], D. Zyss[1], J. Cox[2], G. C. Woods[2], F. Gergely[1]; [1]Cancer Research UK, Cambridge Research Institute, Cambridge, United Kingdom, [2]Cambridge Institute for Medical Research, Cambridge, United Kingdom

Two human centrosomal proteins, CDK5RAP2/Cep215 and Myomegalin share significant sequence homology with Centrosomin, a protein implicated in maintaining centrosome integrity in Drosophila. In addition to their centrosomal localisation, we find that both Myomegalin and CDK5RAP2 are present on the Golgi apparatus. This localisation pattern is strikingly similar to that of the scaffolding protein, AKAP450. Indeed, AKAP450 co-immunoprecipitates with CDK5RAP2, but surprisingly does not bind Myomegalin. We show that CDK5RAP2 targets AKAP450 to the mitotic centrosomes, but the two proteins do not depend on each other for their centrosomal localisation during interphase. CDK5RAP2 has been previously reported to be required for centrosome cohesion. Using RNA interference in Hela and U2OS cells, we show that AKAP450 is important for cohesion. Moreover, the AKAP450-interacting domain of CDK5RAP2 has a dominant-negative effect when overexpressed, as it induces centrosome splitting even when endogenous proteins are present. As we never observed more than 50-60% of cells with split centrosomes at any one time, we suspected that the 'split' phenotype could represent a temporary state of the centrosomes. Indeed, time-lapse analysis of centrin-GFP expressing cells depleted of CDK5RAP2 revealed that centrosomes exhibit dynamic movements. Centrosomes move apart and travel large distances but then they invariably collapse back together. Cumulatively, our data indicates that an interaction between full-length CDK5RAP2 and AKAP450 is important for the cohesion of duplicated centrosomes. In agreement with previous studies, our observations also support the existence of an alternative, highly flexible linker mechanism which is independent of CDK5RAP2. Importantly, homozygous mutations in CDK5RAP2 cause primary microcephaly in humans, yet the reason for this is unknown. The mutation has been reported to cause a protein null phenotype. However, we have immortalised patient-derived cell lines and our preliminary evidence suggests that the CDK5RAP2 mutation may be a hypomorph instead.

1882/B344

Human Centrin's Stability and Their Protein-Protein Interactions.

D. Narvaez, A. Gomez, H. Martir, N. Mendez, B. Pastrana; Chemistry, University of Puerto Rico Mayaguez Campus, Mayaguez, PR

Post-translational modification and calcium binding are key pre-requisites for centrosome duplication and separation. These processes are regulated in healthy tissues and are defective in disease states such as cancer. Centrin is an EF-hand protein that plays both structural and regulatory roles in the centrosome. Two isoforms are presented in this work: human centrin 1 (Hcen1) and human centrin 2 (Hcen2). The mid infrared spectral region of 1700 - 1530 cm-1 was studied to determine the order of events during the thermal perturbation. For Hcen1 the order of events were the following: alpha-helix followed by beta-sheets then glutamate and finally beta-turns while for Hcen2 was: 310-helix followed by aggregation then β-turns, arginine and finally loops. A higher thermal stability was observed for Hcen1 than for Hcen2 and a pre-transition at 1.7 - 4.8 C and the onset of the transition temperature was also observed for Hcen1 at 80.5 - 84 C. Unlike Hcen1, Hcen2 was observed to aggregate at the temperature range of 43 - 58 C. We have also established the relative stability of these proteins by differential scanning calorimetry. Therefore, we were able to establish differences in stability, conformation and dynamics between these closely related calcium binding proteins. Furthermore, this calcium-binding protein interacts at low calcium levels with a novel 1242-amino acid protein known as Sfi1, which contains up to 23 centrin-binding sites. Coupled biophysical, structural, and dynamic analyses of the centrin/Sfi1 complex are essential to the understanding of its biological function. Using an interdisciplinary approach we have determined the conformational changes involved in the centrin-Sfi1p21 complex formation by FT-IR spectroscopy, two dimensional correlation spectroscopy and isothermal titration calorimetry. The binding was exothermic and the thermodynamic data for Hcen1-Sfi1p21 was the following: N 1.33 ± 0.0165, Ka 1.59 x107 ± 2.48 x106 M, ΔH -1.72 x104 ± 301.1 kcal/mol and ΔS -23.8 kcal/mol. Our experiments address key questions underlying the molecular basis of this complex interaction.

1883/B345

Regulation of Polyadenylation of Centrosomal Cyclin B1 mRNA by Cytoplasmic Polyadenylation Binding Protein in Rat Primary Astrocytes.

K. Kim[1], S. Jeon[1], M. Choi[1], S. Shin[1], K. Cho[1], K. Ko[1], C. Shin[2]; [1]Pharmacology, School of Pharmacy, Seoul National University, Seoul, South Korea, [2]Pharmacology, School of Medicine and IBST, Konkuk University, Seoul, South Korea

Cytoplasmic polyadenylation element binding protein (CPEB) is a RNA-binding protein that binds to mRNA containing CPE domain in 3'-untranslated region, and regulates translation of the mRNA via cytoplasmic polyadenylation. CPEB plays a critical role in oocyte maturation, early embryo development, cell cycle regulation, and neuronal plasticity. It is reported that CPEB regulates translation of Cyclin B1 mRNA leading to cell cycle progression in Xenopus laevis. However, there is no clear evidence that CPEB regulates astrocyte cell cycle by regulating polyadenylation of cyclin B1 mRNA in centrosome. In this study, we used sucrose gradient centrifuge to isolate centrosomal fraction and confirmed the localization of CPEB protein and cyclin B1 mRNA in centrosomal fraction obtained from rat primary astrocytes. The length of poly A tail of Cyclin B1 mRNA in both crude astrocyte and purified centrosomal fraction was elongated by proliferative signal such as serum stimulation of cell cycle-arrested astrocytes. Taken together, we propose that CPEB, Cyclin B1 and Aurora A kinase are localized to centrosome and regulate poly A tail elongation of centrosomal Cyclin B1 mRNA, which may be a critical event that regulates proliferation and cell cycle progression of rat primary astrocytes.

1884/B346

The Structure of γ-TuSC and γ-TuSC Rings: Implications for the Mechanism and Regulation of Microtubule Nucleation.

J. M. Kollman[1], R. Choy[1], A. Zelter[2], E. Muller[2], T. Davis[2], D. Agard[1]; [1]Department of Biochemistry & Biophysics and Howard Hughes Medical Institute, UC San Francisco, San Francisco, CA, CA, [2]Department of Biochemistry, University of Washington, Seattle, WA

We have determined the three-dimensional structure of the yeast γ-TuSC, both in isolation and assembled into ring-like complexes, using single particle electron microscopy. These structures provide insight into both the mechanism and the regulation of microtubule nucleation. All eukaryotes have a common mechanism for nucleating microtubules, dependent on γ-tubulin. The γ-tubulin small complex (γ-TuSC) is an evolutionarily conserved heterotetramer, conisisting of two copies of γ-tubulin and one each of Spc97p/Dgrip84/GCP2 and Spc98p/Dgrip91/GCP3. While isolated γ-TuSC is a poor nucleator relative to free γ-tubulin, its activity is greatly enhanced by incorporation into larger complexes and localization at the MTOC. The structure of isolated γ-TuSC is roughly Y-shaped. A combination of direct labeling in EM and in vivo FRET showed that the γ-tubulins are located in lobes at the tips of the Y, with the C-termini of Spc97 and Spc98 near γ-tubulin and their N-termini near the base. The structures of different subpopulations of γ-TuSC indicate flexibility in one arm of the Y, resulting in variation of the relative positions and orientations of the γ-tubulins. In all of the structures the γ-tubulins are in configurations incompatible with the microtubule lattice, one probable explanation for the reduced nucleating capacity of the isolated complex. We have recently made significant progress in understanding the structure of larger oligomers of γ-TuSC. Under conditions that favor the growth of microtubules, γ-TuSC assembles into ring-like structures very similar in appearance to the γ-tubulin ring complex found in animals. We show that these γ-TuSC assemblies can cap microtubule ends. Moreover, by addition of the N-terminal domain of Spc110p, which anchors γ-TuSC to the nuclear face of the spindle pole body, we have induced the organization of γ-TuSC into helical filaments. These filaments are formed of repeating ring structures, composed of laterally interacting γ-TuSCs. Importantly, the structure shows that the γ-tubulins in adjacent γ-TuSCs have the same spacing as laterally interacting tubulin in the microtubule.

1885/B347

Molecular and Functional Characterisation of the Centrosomal Phosphoproteome.

K. Habermann[1], J. Gobom[1], C. Erdmann[2], J. von Kries[2], B. Lange[1]; [1]Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Berlin, Germany, [2]Leibniz-Institut für Molekulare Pharmakologie (FMP), Berlin, Germany

The centrosome of *Drosophila melanogaster* consists of more than 200 proteins that have recently been identified through mass spectrometry (MS) analysis (Mueller et al.). In order to understand the role of the centrosome in cell cycle progression, stress response and signal transduction pathways, we carried out a functional and biochemical characterisation of the identified proteins. Our project aims at identifying centrosomal kinase-substrate interactions and studying the role of phosphorylation events in the regulation of centrosomal function, replication and separation. For this purpose centrosomes were immunopurified from *Drosophila melanogaster* preblastoderm stage embryos. Subsequently, phosphorylated proteins were detected using three complementary approaches: (1) immunoblotting with an antibody against phosphorylated serine and threonine sites labelled 6 proteins, (2) direct in-gel detection of phosphate groups on serine, threonine and tyrosine residues showed 16 proteins. (3) 25 phosphoproteins were identified through MS after enrichment of trypsin-digested phosphopeptides on titanium dioxide columns. Localisation of a subset of these proteins to the centrosome or centrioles was confirmed via TAP- and GFP-tagging and expression in *Drosophila* S2 cells. Currently, we are assigning functional interactions between all identified phosphoproteins and regulatory kinases applying RNAi and subsequent high-content screening immunofluorescence microscopy. S2 cells depleted of phosphorylated proteins and 4 different kinases are analysed with respect to the effects on centrosome number, size, chromosomal aberrations and cell cycle progression. Our preliminary data show that depletion of 6 out of 25 phosphoproteins leads to either a decreased or increased number of mitotic cells as compared to control cells. Furthermore, we found 8 proteins that alter the number of centrosomes per cell and 7 proteins that affect centrosome size. Our results suggest that many of the proteins we identified are directly functioning in signalling pathways that regulate the centrosome cycle and cell cycle, respectively.

1886/B348

Investigation of Novel Centrosome-Associated Candidate Proteins in Human Lymphocytes.

S. H. Macdonald[1], C. Botting[2], A. Long[1], D. Kelleher[1]; [1]Clinical Medicine, Trinity College Dublin, Dublin, Ireland, [2]Biomolecular Sciences, University of St. Andrews, St. Andrews, United Kingdom

The centrosome is a critical integrating centre for many signalling pathways in processes such as cell cycle and migration, as well as a coordinator of cytoskeletal rearrangement during dynamic regulation of cell shape. In our previously- established model of lymphocyte migration, the centrosome consistently reoriented to the 'neck' of the polarised cell, behind the nucleus, whilst in a model of antigen presentation, it reoriented to a location in close proximity to the T cell- antigen presenting cell (APC) interface. It is likely that the centrosome and associated signalling elements perform a significant role in these two processes, which are fundamental to normal immune surveillance. To identify potential novel regulators of centrosome function, centrosomes isolated from the human T lymphoma cell line,

HuT78, were analysed using a proteomics approach. In addition to known centrosome components, several proteins were detected that are implicated in processes such as central nervous system (CNS) development and neuronal migration, such as Neuron Navigator-2 (NAV2) and Discs Large homolog (Dlg). Candidate proteins were chosen for further investigation that were known to be involved in structure/ migration processes, but had not been previously studied in lymphocyte function. Of these, several were confirmed to be localised to the centrosome, by dual immunofluorescent staining in conjunction with antibodies to centrosomal marker proteins. Further, in migrating HuT78 cells, many of these candidate proteins were found to localise to distinct compartments, such as the leading edge, or the neck and tail region of polarised cells. This differential trafficking may indicate previously unknown roles in regulation of cell morphology, particularly in the context of outside-in signalling. Certain proteins were also found to contain RNA- recognition motifs, and may represent a further mechanism of control for localised expression during cell migration. Additionally, many of the proteins detected in our analysis are mutated or aberrantly expressed in a number of cancer types, and may potentially give insight into novel mechanisms contributing to transformation and metastasis.

1887/B349

Modulation of Gamma-Tubulin Localization in Metastatic Breast Tumor Cell Lines.

E. Cho, R. Whipple-Bettes, M. A. Matrone, E. Balzer, S. S. Martin; University of Maryland at Baltimore School of Medicine, Baltimore, MD

γ-tubulin is the necessary protein for microtubule nucleation to occur in mammalian cells. In interphase human cells, γ-tubulin is thought to be found exclusively at the centrosome where nucleation of microtubules (MTs) is initiated. Disruption of MTs is an active target in cancer research to treat primary tumors. However, there is little evaluation of the effects of these drugs on metastasis. In this study we examine the role of γ-tubulin ring complex (γ-TuRC) proteins in metastatic breast cancer to elucidate the role of MTs in metastatic breast cancer. Previous studies have reported evidence of γ-tubulin may also exist outside of the centrosome, but the reasons for this remain unclear. Our current studies show a modulation of γ-tubulin localization in a panel of normal and tumorigenic human breast cell lines of increasing metastatic potential, when gauged by indirect immunofluorescence. Image analysis of fluorescence images reveals at least a four-fold increase of γ-tubulin in highly invasive breast cancer cells lines over a non-tumorigenic and non-invasive cell lines. However, total levels of protein expression for γ-tubulin remained at similar levels for all cell lines by SDS-PAGE analysis. An insoluble pool of γ-tubulin localized outside the centrosome supports the possibility of microtubule nucleation in the cytosol, particularly along pre-existing MTs. The possibility of microtubule nucleation along pre-existing MTs has major implications on current breast cancer therapies that target microtubule stabilization, especially in light of new microtubule-based membrane protrusions (microtentacles) that have been described in breast tumor cells. Drugs which inhibit primary tumor growth are often ineffective against disseminated metastatic tumor cells that often remain dormant for years before progression into a secondary tumor. Thus, drugs like paclitaxel may be ineffective against metastatic cells, and potentially promote counterproductive microtubule growth in tumor cells with an abundance of cytoplasmic γ-tubulin. Further investigation of the role of γ-tubulin and other γ-TuRC proteins could reveal more effective ways to target the cytoskeleton of metastatic tumor cells.

1888/B350

The Phosphoproteome and Phospho-Regulation of the Yeast Centrosome.

J. M. Keck[1], C. C. Wong[2], M. Jones[1], E. Holinger[1], J. R. Yates[2], M. Winey[1]; [1]University of Colorado, Boulder, CO, [2]The Scripps Research Institute, La Jolla, CA

The budding yeast centrosome (known as the spindle pole body or SPB) functions to nucleate microtubules in the nucleus and cytoplasm. The SPB duplicates early in the cell cycle and forms a bipolar mitotic spindle to capture and segregate chromosomes during cell division. A few studies have shown that protein phosphorylation can contribute to SPB assembly and SPB function in microtubule nucleation. Our goal is to greatly expand the study of centrosome phosphorylation by providing a comprehensive inventory of phosphorylation events found on all 18 core SPB proteins. Using a novel method to isolate large quantities of intact SPB complexes, we are able to perform mass spectrometry analysis to identify numerous phosphorylation sites on proteins within the SPB. This information will be used to uncover regulatory mechanisms of SPB assembly and function and to assign known regulators, such as Cdc28 and Mps1 kinase, to particular phosphorylation events. To date, we have identified 204 novel phosphorylation sites on SPB proteins. Currently we are mapping phosphorylation sites from cells arrested in the cell cycle at G1 phase and mitosis, to correlate cell cycle timing of particular phosphorylation events with function. We are prioritizing the analysis of specific sites according to both degree of conservation of the residue and cell cycle regulation. We also demonstrate the importance of SPB phosphorylation by mutating a single phosphorylated residue identified in Spc29 to a nonphosphorylatable alanine. This mutation leads to severe defects in SPB assembly. The phosphoproteome project will greatly expand our knowledge of phosphorylation at the SPB and overall understanding of phospho-regulation of centrosomes.

1889/B351

A Role for IFT88 in Maintaining Centrosome Integrity, Microtubules and Mitotic Spindle Organization and Spindle Orientation in Cultured Cells and Zebrafish Embryos.

B. Delaval, A. Bright, C. Powers, S. Doxsey; Molecular Medicine, UMass Medical School, Worcester, MA

Centrosomes are essential for the organization and positioning of the mitotic spindle. In non-cycling cells, the centrosome contributes to cilia assembly. Cilia proteins essential for cilia assembly in non cycling cells such as intraflagellar transport proteins (IFTs), localize to centrosomes in non ciliated, mitotic cells. Cilia/centrosome proteins have been implicated in human ciliopathies. Functions disrupted in these disorders--cell cycle regulation, orientation of cell division and cilia formation--appear to be centrosome-based. To address the roles of centrosomes and mitosis in ciliopathies, we examined a well-characterized model of this disorder, IFT88 depletion. Using zebrafish embryos and cultured cells we show that IFT88 depletion disrupts centrosomes, microtubules and mitotic spindles. More specifically, IFT88 depletion leads to defects in centrosomes/spindle poles and an increase in phospho-histone 3 positive, “prometaphase-like” mitoses. Cells with such defects are delayed in mitosis as shown by time-lapse imaging. To understand the mechanism by which IFT88 depletion induces defects in spindle organization, we examined spindle microtubules and molecules that modulate them. Spindle poles were unfocused when examined by gamma tubulin staining and gamma tubulin itself was reduced. Spindle microtubules were disengaged from spindle poles or reduced in stability as shown by decreased staining for acetylated tubulin. Astral microtubules were either absent or reduced in length and did not

contact the plasma membrane, a requirement for orientation of the spindle. In fact, IFT88-depleted cells showed misoriented spindles, a phenotype thought to lead to cyst formation in kidneys and other organs. Consistent with astral microtubule loss and spindle misorientation was a dramatic reduction of the spindle pole-associated fraction of the microtubule plus end binding protein EB1, a protein required for astral microtubule formation and spindle orientation. IFT88 immunoprecipitated with EB1 in mitotic cell lysates. This work uncovers a mitotic function for IFT88 and suggests that cilia proteins contribute to ciliopathies through disruption of centrosome integrity, microtubule organization, and spindle orientation.

1890/B352

The Hydrolethalus Syndrome Protein HYLS-1 Links Core Centriole Structure to Cilia Formation.

A. Dammermann[1], H. Pemble[1], B. Mitchell[2], I. McLeod[3], J. R. Yates III[3], C. Kintner[2], A. B. Desai[1], K. Oegema[1]; [1]Ludwig Institute for Cancer Research, UCSD, La Jolla, CA, [2]The Salk Institute for Biological Studies, La Jolla, CA, [3]The Scripps Research Institute Institute, La Jolla, CA

A key function of centrioles is to template the formation of cilia; however, the proteins involved in this process have remained largely unknown. HYLS-1 was originally identified as a protein mutated in hydrolethalus syndrome, a perinatal lethal human developmental disorder. We show that HYLS-1 stably incorporates into centrioles during their assembly in a manner dependent on SAS-4, a core centriolar structural protein with which it physically interacts. Unlike SAS-4, HYLS-1 is dispensable for centrosome duplication and function. Instead, HYLS-1 is specifically required for cilia formation in both C. elegans and Xenopus embryos. The mutation found in hydrolethalus syndrome does not affect HYLS-1 localization, but impairs its function in cilia assembly. HYLS-1 thus forms part of the critical link between core centriole structure and cilia formation.

Mitosis and Meiosis III (1891 – 1919)

1891/B354

N-Terminal Coiled-Coil of Ndel1 Binds Dynein and Is Required for Dynein-Dependent Spindle Pole Focusing in Xenopus Egg Extracts.

E. Zylkiewicz[1,2], M. A. Kijanska[1,2], W. Choi[2], U. Derewenda[2], Z. S. Derewenda[2], P. Stukenberg[1]; [1]Biochemistry & Molecular Genetics, University of Virginia, Charlottesville, VA, [2]Molecular Physiology & Biological Physics, University of Virginia, Charlottesville, VA

Cytoplasmic dynein is a major minus-end directed microtubule motor complex, and is required for a variety of cellular functions including intracellular transport, nuclear and neuronal migration, and regulation of the mitotic spindle. How cytoplasmic dynein is regulated to perform so many functions is a major unsolved question. In vertebrates Nde1 and Ndel1 are encoded by two homologous genes, and have been implicated in a subset of dynein-related processes including nuclear migration and targeting dynein to kinetochores. Both Nde1 and Ndel1 contain long coiled-coil N-termini that bind another dynein regulator Lis1, followed by a C-terminal domain that targets Ndel1 to kinetochores and binds dynein. In Xenopus extracts dynein is required during spindle morphogenesis where it focuses the minus ends of microtubules to form spindle poles. We found that Xenopus Nde1 and Ndel1 are required for this function of dynein. Xenopus egg extracts depleted of Nde1 and Ndel1 nucleate microtubules normally, but are unable to focus them into Ran asters. We have rescued depleted extracts with mutants of Ndel1 to map the minimal domains required for aster formation. The N-terminal coiled-coil domain of Ndel1 is necessary and sufficient for aster formation and the mapped dynein interaction domain on the C-terminus is not required. We have identified a second dynein interaction domain in the N-terminus of Ndel1. Our data demonstrate that Nde1/Ndel1 have redundant roles to regulate dynein function in spindle morphogenesis and that this requires the N-terminal part of Nde1/Ndel1 to interact with dynein. We argue that the C-terminus inhibits this interaction unless the proper signals are produced.

1892/B355

Eg5/Dynein Antagonism Requires Overlapping Centrosomal Microtubules.

N. Ferenz[1], R. Paul[2], A. Mogilner[2], P. Wadsworth[1]; [1]University of Massachusetts, Amherst, MA, [2]University of California, Davis, CA

Assembly of bipolar mitotic spindles requires the antagonistic activities of various molecular motor proteins. In mammalian cells, for example, monastrol-induced monopolar arrays reorganize into bipolar spindles following dynein inhibition. Similarly, Eg5- and HSET-inhibited cells bypass the monopolar phenotype altogether. Because antagonism between plus-end and minus-end directed motors conceptually involves antiparallel microtubules, we tested the contribution of centrosomal arrays to Eg5/dynein antagonism in mammalian cells expressing GFP-tubulin. To do this, we employed a nocodazole washout assay in which cells were first treated with nocodazole to disassemble microtubules, subsequently treated to inhibit Eg5 and/or dynein, and then washed with nocodazole-free medium to initiate spindle assembly. Prior to nocodazole removal, the centrosomes could be found either adjacent to, or spatially separated from, the chromosomal region (proximal and distal centrosomes, respectively). This variation in the position of centrosomes made it possible to examine antagonism during spindle assembly with and without the influence of their arrays. Our data demonstrate that when proximal centrosomes are initially located close to one another (<6μm), antagonism between Eg5 and dynein occurs (i.e., bipolar spindles form when both motors are inhibited, whereas monopolar arrays form following Eg5 inhibition alone). However, when proximal centrosomes are initially distant (>6μm apart), monastrol treatment fails to collapse spindles, thereby eliminating the possibility of antagonism. Additionally, in cells containing distal centrosomes, bipolar arrays form around chromosomes independently of dynein or Eg5 activity, again abolishing the opportunity for antagonism. In silico simulations of two centrosomal asters interacting with chromosomes and each other reflect our live cell analyses. These simulations illustrate stable monopolar and bipolar configurations, depending on the initial inter-centrosomal distance, when Eg5 is inhibited and stable bipolar spindles when both Eg5 and dynein are inhibited. These results suggest that Eg5/dynein antagonism depends on the presence, and sufficient overlap, of centrosomal microtubules.

1893/B356

Mechanisms of DAM1 Complex Motility with the Ends of Shortening Microtubules.

V. Volkov[1,2], E. L. Grishchuk[1,3], I. Spiridonov[1,2], A. K. Efremov[1,2], N. Gudimchuk[4], S. Westermann[5], D. Drubin[6], G. Barnes[6], J. McIntosh[1], F. I. Ataullakhanov[2,4,7]; [1]Molecular, Cellular and Developmental Biology, University of Colorado, Boulder, CO, [2]National Research Centre for Hematology, Moscow, Russian Federation, [3]Institute of General Pathology and Pathophysiology, Moscow, Russian Federation, [4]Physics Department, Moscow State University, Moscow, Russian Federation, [5]Research Institute of Molecular Pathology, Vienna, Austria, [6]Molecular and Cell Biology, University of California, Berkeley, CA, [7]Center for Theoretical Problems of Physicochemical Pharmacology, Russian Academy of Sciences, Moscow, Russian Federation

Mitotic chromosomes segregate at the ends of shortening spindle microtubules. In budding yeast, the Dam1 multiprotein complex supports this dynamic attachment, thereby contributing to accurate chromosome segregation. Purified Dam1 will track the end of a depolymerizing MT and can couple it to microbead transport in vitro. The processivity of such motions has been thought to depend on rings that the Dam1 complex can form around MTs, but the possibility that alternative coupling geometries contribute to these motilities has not been evaluated experimentally. We investigated the tracking and coupling properties of rings and non-ring Dam1 assemblies in vitro using quantitative fluorescent light microscopy. We demonstrate that non-encircling assemblies of Dam1, as well as rings, can track microtubule ends. The kinetic properties of these two populations are quite different: smaller complexes retard microtubule shortening less than the rings do, and they diffuse faster than rings on microtubule lattice. We also demonstrate that both rings and non-encircling complexes enable processive movement of microbeads, though by different mechanisms: in the presence of rings beads slide, while in the absence of rings they roll along microtubule. We suggest that different oligomeric forms of Dam1 may contribute to mitotic chromosome motion. To better understand the mechanism of ring motility, we have investigated the force transducing properties of wild-type Dam1 and the Dam1-19 mutant (truncated C-terminus of Dam1p) using the laser trapping of Dam1-coated beads. Our results demonstrate that the mutant complex is a less efficient force transducing coupler than wild type complex, suggesting the importance of strong binding between the C-terminal tail of Dam1 protein and tubulin. These and other results, such as the bead's irregular oscillations during forced motion, suggest that Dam1 rings move by a "forced walk" mechanism.

1894/B357

A Role for Lamin-B in Antagonizing Eg5 Activity during Spindle Assembly in Xenopus Egg Extracts.

B. Goodman[1,2], W. Channels[3], P. Iglesias[3], Y. Zheng[1,2]; [1]Embryology, Carnegie, Baltimore, MD, [2]Biology, Johns Hopkins, Baltimore, MD, [3]Electrical and Computer Engineering, Johns Hopkins, Baltimore, MD

The mitotic spindle is composed of microtubules, motor proteins, spindle assembly factors, and the recently defined membranous spindle matrix [1]. Xenopus laevis egg extract is one system used to study mitotic spindle assembly in vitro. Both sperm and the GTP-bound form of RanGTPase (RanGTP) can be used to induce spindle formation in Xenopus egg extract. However, it is difficult to visualize spindle bipolarization live because it is difficult to follow the spindle poles in these relatively slow reactions. Previously, we showed that Aurora-A coated magnetic beads function as microtubule organizing centers (MTOC) in Xenopus egg extracts and induce rapid spindle assembly [2]. Since the beads are auto-fluorescent, we are able to track spindle bipolarization and pole separation during spindle assembly induced by Aurora-A beads. Using Aurora-A-bead-based assays, we show that spindle bipolarization is driven by the separation of Aurora-A beads. The bead separation rate can be reduced by monastrol, a drug that inhibits Eg5 activity [3]. Additionally, with the Aurora-A bead assay, we can track MTOCs as individual beads, allowing us to quantify MTOC clustering. We found that bead clustering promoted by monastrol could be reversed by depletion of lamin B3, a major component of the spindle matrix [1]. These results indicate that one function of Lamin-B3 during spindle assembly is to antagonize Eg5 through an as-yet-unknown mechanism. 1. Tsai et al. A mitotic lamin B matrix induced by RanGTP required for spindle assembly. Science (2006) vol. 311 (5769) pp. 1887-93. 2. Tsai et al. Aurora A kinase-coated beads function as microtubule-organizing centers and enhance RanGTP-induced spindle assembly. Curr Biol (2005) vol. 15 (23) pp. 2156-63. 3. Mayer et al. Small molecule inhibitor of mitotic spindle bipolarity identified in a phenotype-based screen. Science (1999) vol. 286 (5441) pp. 971-4.

1895/B358

A Rangtp Gradient Is Involved in the Spatial Organization of Microtubules in Somatic Mitotic Cells.

J. Zonis, A. Wilde; Molecular Genetics, University of Toronto, Toronto, ON, Canada

Studies suggest that spindle formation in mitotic cells requires a positional cue from the chromosomes. A good candidate for this cue is the small GTPase Ran. RanGTP is generated at chromosomes by its guanine nucleotide exchange factor RCC1. In the GTP bound form Ran leads to the activation of spindle assembly factors. A gradient of RanGTP has been visualized around mitotic chromosomes, but the significance of this gradient has never been demonstrated *In Vivo* and has been predicted to be only of importance in large embryonic cells but not in somatic cells. To determine whether RanGTP provides spatial information within somatic cells for microtubule (MT) organization and spindle assembly, we created an ectopic site of RanGTP generation at the plasma membrane (PM) in HeLa cells. We observed the formation of organized microtubule arrays at the PM in ~ 30% of these cells. In contrast, no PM associated MT arrays were seen in cells where there was increased cytosolic or chromosomal generation of RanGTP. γ-tubulin localized to the organized center of these arrays at the PM, suggesting that + ends of the MTs extended into the cytoplasm. In addition, known Ran effectors in spindle formation, such as TPX2 and Aurora A, localized to these structures. These data suggest that the localized elevation of RanGTP levels in mitotic cells induces localized generation and organization of MTs. To determine if the site of RanGTP generation acts as a spatial cue towards which MTs grow, we generated RanGTP at the PM of tsBN2 cells grown at the non permissive (NP) temperature of 39.5^0 C, a temperature where endogenous, chromosomal RCC1 is rapidly degraded. In mitotic tsBN2 cells at the NP temperature bipolar spindles did not form. Instead a rosette formed with DNA around the rim of the rosette and MTs growing from centrosomes at the center and terminating at the DNA. In contrast, in tsBN2 cells at the NP temperature where the only site of RanGTP generation was at the plasma membrane, microtubules grew from the centrosomes through the DNA towards the plasma membrane. These findings suggest that in mitotic somatic cells RanGTP might serve as a positional cue which organizes microtubules and directs their growth.

1896/B359

The Localized Production of RanGTP at the Chromatin-Cytoplasm Interface Is Sufficient and Required for Bipolar Mitotic Spindle Assembly in Xenopus Laevis Egg Extracts.

P. Kalab[1,2], D. R. Halpin[2], R. Heald[2], K. Weis[2]; [1]Laboratory of Cellular and Molecular Biology, National Cancer Institute, NIH, Bethesda, MD, [2]Department of Molecular and Cell Biology, University of California at Berkeley, Berkeley, CA

The essential role of the GTPase Ran in mitotic spindle assembly is thought to depend on the existence of RanGTP gradient surrounding chromosomes, which is generated by the binding of RCC1, the guanine nucleotide exchange factor for Ran, to chromatin, and the cytoplasmic localization of RanGAP. The RanGTP gradient locally releases spindle assembly factors (SAFs) from their inhibitory complexes with importin β, thus directing mitotic spindle assembly towards chromosomes. To analyze how such local regulation is achieved, we observed mitotic spindle assembly in *Xenopus laevis* egg extracts using fluorescence lifetime microscopy (FLIM) of improved fluorescence resonance energy transfer (FRET)-based molecular sensors for Ran. This approach allowed us to indirectly visualize RanGTP, and observe the release of importin β cargos, while following chromatin and microtubule dynamics in the same mitotic extract by time-lapse quantitative microscopy. Although the titration of 5-20μM RanGTP into the extracts released importin β cargos and induced microtubule aster formation, cargos were never fully released in chromatin-free cytoplasm, even in the presence of a large excess of free RanGTP. In contrast, the concentration of free cargos present around mitotic spindle chromosomes exceeded that driven by >20μM RanGTP added to the cytoplasm. When beads coated with active RCC1 were added to the extracts, gradients of free importin β cargos formed around them, similar to chromosomes, and mostly bipolar mitotic spindle-like structures formed. In the *X.laevis* egg extract system, the RanGTP gradient is therefore a dominant chromatin activity sufficient to direct bipolar mitotic spindle assembly. Furthermore, our results show that full activation of the cytoplasmic spindle assembly pathways requires reaction conditions present at the chromatin-cytoplasm interface, providing a robust spatial clue for mitotic spindle assembly.

1897/B360

A Small Molecule Inhibitor of the Ran/Importin-β Interaction.

S. L. Bird, J. Soderholm, R. Heald, K. Weis; Molecular & Cell Biology, University of California Berkeley, Berkeley, CA

The small GTPase Ran and its binding partner importin-β are involved in the control of many cellular functions. In interphase, the Ran pathway plays an essential role in controlling nucleocytoplasmic transport. Additionally, in mitosis, Ran directs the chromosome mediated pathway of mitotic spindle assembly. The essential nature of Ran's role in interphase makes it challenging to study the Ran pathway in mitosis using methods such as genetics or RNAi. In order to overcome this problem, we used a high-throughput FRET based screen to identify a small molecule inhibitor of the Ran/importin-β interaction which we call Importazole (IPZ). Here, we demonstrate that IPZ disrupts importin-β mediated nuclear import both in vitro and in vivo, but has no effect on nucleocytoplasmic transport processes not involving importin-β. In addition, we show the ability of IPZ to disrupt mitotic spindle assembly in a concentration dependent manner, and to produce a phenotype in mitotic HeLa cells not previously associated with the Ran pathway. We conclude that IPZ will serve as an exceedingly valuable tool for future study of the Ran pathway in mitosis.

1898/B361

Reconstitution of Mitotic Spindle Assembly in Xenopus Egg Extracts with Single RCC1-Coated Beads.

D. R. Halpin[1], P. Kalab[2], J. Wang[1], K. Weis[1], R. Heald[1]; [1]Molecular & Cell Biology, UC Berkeley, Berkeley, Ca, CA, [2]Cellular and Molecular Biology, National Cancer Institute, Betheseda, MD

Mitotic chromosomes generate biochemical signals that promote microtubule polymerization and spindle assembly. Chromatin itself, formed on plasmid-DNA coated beads, is sufficient to induce spindle assembly in Xenopus egg extracts, but the molecular mechanisms underlying this phenomenon are poorly understood. One component of the chromatin-driven pathway is the GTPase Ran, which stimulates microtubule polymerization when bound to GTP. Chromosomally localized RCC1, the guanine nucleotide exchange factor for Ran, and its cytosolic GTPase activating protein, Ran GAP, generate a gradient of RanGTP surrounding mitotic chromosomes. In order to investigate the sufficiency of this pathway for spindle assembly, we developed a novel bead substrate. Individual, porous microbeads 10 microns in diameter were coated with chromatin or just the RCC1 protein. Both types of beads generated bipolar or multipolar spindles of similar dimensions in egg extracts. RCC1 beads induced bipolar and multipolar microtubule structures within ten minutes, although the overall efficiency was lower than for chromatin beads (30% vs. 75%, respectively). The percentage of bipolar spindles formed around RCC1 beads increased dramatically upon addition of wild-type Ran, indicating that localized hyperactivation of the RanGTP pathway is sufficient for spindle assembly. Like chromatin bead spindles, RCC1-induced spindles exhibited poleward microtubule flux, and treatment with the Kinesin-5 inhibitor monastrol prevented spindle formation. However, unlike chromatin beads, RCC1 beads oscillated within spindle structures. RanGTP-sensitive FRET probes and biochemical assays are being used to directly compare RCC1 activity levels on RCC1 beads to chromatin beads. We are in the process of coupling other chromatin-associated factors to beads, including chromokinesins and the kinases Polo and Aurora B, both individually and in combination with RCC1. These studies will elucidate the chromatin-mediated pathways essential for spindle assembly and form the basis for complete spindle reconstitution.

1899/B362

Spindle Assembly in the Absence of a RanGTP Gradient.

A. Groen[1,2], J. Gatlin[1,3], T. Mitchison[1,2], E. D. Salmon[1,3], T. J. Maresca[1,3]; [1]Cell Division Group, Marine Biological Laboratory, Woods Hole, MA, [2]Systems Biology, Harvard Medical School, Boston, MA, [3]Biology, University of North Carolina, Chapel Hill, NC

During cell division, the GTP-bound state of the small GTPase Ran is promoted around chromosomes by its chromatin-associated GEF. This leads to the formation of a RanGTP gradient around DNA that induces a localized activation of spindle assembly factors (SAFs). While it is clear that the generation of GTP-bound Ran is required for spindle assembly, it is unclear whether a gradient of RanGTP around chromatin is essential. Here we demonstrate that spindles can assemble in the absence of a RanGTP gradient. The RanGTP gradient was removed by the

simultaneous addition of two Ran mutants to spindle assembly reactions in X. laevis egg extracts. RanT4N (T), which mimics the nucleotide-free state of Ran and binds with high affinity to the GEF, was used to shut off chromatin-localized RanGTP generation and RanQ69L (Q), which is constitutively GTP-bound, was added to activate all Ran-regulated SAFs independent of any spatial cues. Surprisingly, Q/T reactions supported spindle assembly around replicated sperm nuclei but not around chromatin beads. Since chromatin beads lack centromeres and kinetochores we next investigated the contribution of the chromosomal passenger complex (CPC) to RanGTP gradient independent spindle assembly. Interestingly, Q/T reactions no longer supported sperm spindle assembly following immunodepletion of INCENP or chemical inhibition of Aurora B. Conversely, hybrid bead clusters consisting of both INCENP-bound beads and chromatin beads supported the local assembly of MT arrays and bipolar spindles under Q/T conditions. Thus, we conclude that two localized signals are generated around DNA to direct proper spindle assembly: one mediated by kinetochores the other by chromatin. We hypothesize that the kinetochore signal is dominated by CPC activity and the chromatin signal is predominantly RanGTP-mediated.

1900/B363

Investigating the Role of Membrane Trafficking GTPases in Spindle Assembly and Function.

B. Riggs, J. Davis, R. Heald; Molecular and Cell Biology, University of California Berkeley, Berkeley, CA

The mitotic spindle is crucial for the proper segregation of chromosomes during cell division, but the molecular mechanisms underlying spindle assembly and function are poorly understood. Recent evidence indicates a potential role for membrane-associated proteins, since several microtubule associated proteins (MAPs) necessary for normal spindle function have been found to associate with membrane vesicles. Furthermore, unlike crude *Xenopus laevis* egg extracts generated by low speed centrifugation, high speed supernatants lacking membranes do not support bipolar spindle assembly *in vitro*. We found that Brefeldin A (BFA), a small molecule inhibitor of Golgi vesicle trafficking, disrupted proper spindle formation and chromosome segregation when added to crude extract prior to spindle assembly. To test the specificity of this effect, mutant versions of Arf1, the putative target of BFA, were added to the extract and effects on spindle assembly and chromosome segregation evaluated. Arf1(Q71L), a constitutively active mutant, had only minor effects on spindle assembly with a 10% reduction in normal structures compared to controls, but strongly inhibited chromosome segregation when added just prior to the start of anaphase. In contrast, addition of the inactive mutant Arf1(T31N) that inhibits generation of ArfGTP did not impair chromosome segregation. We are similarly evaluating other GTPases involved in intracellular membrane dynamics for effects on spindle assembly and chromosome segregation including Sar1 which functions in vesicle trafficking in the endoplasmic reticulum. We hypothesize that active regulation of membrane vesicles is required for proper spindle assembly and function during mitosis.

1901/B364

BubR1 Kinase Facilitates Kinetochore-Microtubule Attachments Independently from Its Checkpoint Function.

Z. Rahmani[1], M. E. Gagou[2], C. Lefebvre[2], D. Emre[1], R. Karess[1,2]; [1]CNRS UMR 7592, Institut Jacques Monod, Paris cedex 05, France, [2]Centre de Génétique Moléculaire, CNRS UPR 2167, Gif-sur-Yvette, France

BubR1 is a conserved component required both for the checkpoint and for establishing proper connections betwen kinetochores and the spindle. The role of the kinase domain of BubR1 (which is absent in yeast and worms) is controversial. Therefore, we generated and analyzed a *bubR1*-kinase dead (*bubR1-KD*) mutant in *Drosophila*. We found that *bubR1-KD* flies were viable and fertile. Moreover, *bubR1-KD* cells were still checkpoint competent, with a very low level of aneuploidy, and displayed a robust mitotic arrest in response to colchicine treatment. However, prometaphase chromosome alignement was delayed, kinetochore fibers were thin, and spindle length was unstable, indicating a defect in generating or maintaining kinetochore-microtubule attachments. To make a potentially "checkpoint dead" bubR1 mutant, the N-terminal KEN box, known to be required in Mad3 (the BubR1 homologue in yeast) for binding to Cdc20, was mutated to alanines (*bubR1-KEN*$^{-}$). *bubR1-KEN*$^{-}$ flies were viable and fertile, and mutant cells showed no obvious defects in spindle assembly or mitotic progression, and a very low level of aneuploidy. By contrast to the *bubR1-KD* mutant, *bubR1-KEN*$^{-}$ cells were entirely checkpoint defective and failed to accumulate in mitosis following colchicine treatment. Altogether, these results argue that the kinase activity of BubR1 is required for promoting kinetochore attachment but is dispensable for the checkpoint whereas the "checkpoint activity" of BubR1 is not required for kinetochore capture, but is of course essential to the checkpoint. We conclude that the checkpoint and kinetochore-microtubule capture functions of BubR1 are separable and independent. The *bubR1-KEN*$^{-}$ mutant flies allow us to study genetically for the first time the "pure" checkpoint function for BubR1.

1902/B365

Kinesin-6 Klp9p Is Anchored to Microtubule Bundler Ase1p at the Spindle Midzone in a Phosphorylation-Regulated Manner for Proper Anaphase B.

C. Fu[1], J. Ward[2], I. Loiodice[1,3], F. Nedelec[2], P. Tran[1,3]; [1]Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA, [2]Cell Biology and Biophysics, EMBL, Heidelberg, Germany, [3]Cell Biology, Institut Curie - CNRS, Paris, France

The spindle midzone - composed of antiparallel interdigitating microtubules (MTs), microtubule-associated proteins (MAPs), and microtubule motors - is the cytoskeletal structure responsible for MT organization and sliding during spindle elongation at anaphase B. In general, MAPs stabilize the bipolar structure of the midzone and motors produce the motive force for sliding. How motors and MAPs are coordinated for proper anaphase B is not well understood. Here, we show that the novel fission yeast mitotic kinesin klp9p binds to the MT antiparallel bundler ase1p at the midzone at the onset of anaphase B. This physical interaction depends upon the phosphorylation states of both klp9p and ase1p. The cyclin-dependent kinase cdc2p and its antagonist phosphatase clp1p directly phosphorylates and dephosphorylates klp9p and ase1p during mitosis to control the positioning and timing of klp9p-ase1p interaction. Failure of klp9p-ase1p binding leads to a decreased velocity of spindle elongation. Computer modeling suggests that klp9p antiparallel interaction with ase1p produces the most efficient MT sliding. Our finding implicates a phosphorylation switch that controls the spatial-temporal interactions of motors and MAPs for proper anaphase B.

1903/B366

Interplay of Microtubule Dynamics and Sliding during Bipolar Spindle Formation in Mammalian Cells.

S. Kollu, D. Compton; Biochemistry, Dartmouth Medical School, Hanover, NH

The organization of microtubules into bipolar spindles is essential for accurate chromosome segregation during mitosis. Perturbation of various mitotic proteins disrupts bipolar spindle assembly resulting in monopolar spindles. Notably, members of the kinesin-5 family (i.e. Eg5) are essential for spindle bipolarity and clinical trials are currently evaluating Eg5 inhibitors for anti-tumor activity. However, in mammalian somatic cells, Eg5 activity is required only to establish spindle bipolarity and is dispensable once spindle bipolarity is achieved. Here, we examine the requirements for bipolar spindle maintenance in the absence of Eg5 activity in human cultured cells. We show that mitotic cells deficient in proteins that regulate microtubule dynamics fail to maintain spindle bipolarity without Eg5 activity. The collapse of bipolar spindles under these conditions is driven by activities that focus microtubule minus ends at spindle poles. Furthermore, treatments that increase microtubule dynamics reduce cell sensitivity to inhibition of Eg5 activity. These data demonstrate that bipolar spindle assembly in mammalian cells involves the functional interplay of microtubule dynamics and sliding activities. The anti-parallel microtubule sliding activity of Eg5 is essential for centrosome separation in early phases of mitosis when microtubule dynamics is low, and becomes dispensable when dynamics sharply increase as cells transit to metaphase. These data reveal routes whereby alterations of microtubule dynamics may generate resistance to Eg5 inhibitors.

1904/B367

Proteomic and Functional Characterization of Mitotic Microtubule Associated Proteins Identifies a Novel Kinesin-Like Protein Necessary for Bipolar Spindle Assembly.

J. Torres[1,2], M. Summers[1,2], L. Belmont[2], L. Corson[2], M. Brauer[2], P. Jackson[1,2]; [1]Genentech, South San Francisco, CA, [2]Stanford University School of Medicine, Palo Alto, CA

During mitosis chromosome segregation relies on the proper formation and stabilization of the mitotic spindle. Although many proteins have been identified that contribute to these processes, presumably many others are involved. Therefore, we sought to identify novel proteins involved in mitotic spindle formation. To this end, mitotic HeLa cell extracts were used for in extracto microtubule polymerization reactions and proteins co-pelleting with microtubules were identified by mass spectrometry. We generated a Dharmacon siRNA library directed against 600 microtubule binding proteins for use in various high throughput genetic screens, including mitotic spindle assembly, mitotic arrest, mitotic bypass, and apoptosis. Among the positive hits from these screens was a novel kinesin-like protein termed KifX. KifX is ubiquitously expressed at low levels among all tissues examined. KifX localizes to the centrosome and more specifically to the centrioles in a cell cycle specific manner, from prophase to anaphase. Depletion of KifX resulted in multipolar spindle formation, however no centrosome over-duplication was observed. KifX-depleted cells exhibit pericentriolar material (PCM) fragmentation, indicated by multiple Pericentrin and gamma-Tubulin foci, but only two centrosomes by Centrin staining. Moreover, this defect can be rescued by inhibition of the mitotic kinesin Eg5 prior to mitotic entry. This implicates KifX in a novel early mitotic mechanism, post centrosome separation, which is required to keep the PCM intact. Localization of spindle focusing activities to spindle poles is not affected in KifX-depleted cells as these factors localized to ectopic spindles. Live time-lapse microscopy showed that KifX-depleted cells enter mitosis but fail to align chromosomes at the metaphase plate. These cells do not exit mitosis, condense their chromatin, and apoptose in mitosis. Caspase activation studies have determined that KifX-depleted cells have increased caspase 3/9 activity along with decreased cell viability. KifX represents a novel factor in spindle formation and may help to elucidate the requirements for triggering mitotic apoptosis, sought after in antimitotic therapeutics.

1905/B368

Microtubule Cross-Linking and Sliding Abilities of Kinesin-14 HSET/XCTK2 Are Important for Spindle Assembly and Morphology.

S. Cai[2], L. N. Weaver[1], S. Ems-McClung[3], C. Walczak[3]; [1]Biology, Indiana University, Bloomington, IN, [2]Biochemistry Program, Indiana University, Bloomington, IN, [3]Medical Science Program, Indiana University, Bloomington, IN

Kinesins are molecular motors that utilize ATP hydrolysis to move in a unidirectional manner on microtubules (MTs). The Kinesin-14 family members are minus end directed motors that cross-link and slide MTs, making them key players in spindle assembly. We previously showed that the small GTPase Ran regulates the *Xenopus* Kinesin-14 XCTK2 via the bipartite nuclear localization signal (NLS) in the tail of XCTK2. Importin α/β bind to this signal, which sequesters XCTK2 in the nucleus and inhibits MT binding and cross-linking. Upon nuclear envelope breakdown, XCTK2 is released so that it may cross-link MTs to organize the mitotic spindle. Here we show that overexpression of the human Kinesin-14 HSET in HeLa cells results in an increase in spindle length, while HSET RNAi knockdown results in shorter spindles. In addition, overexpression of HSET-N593K, an HSET mutant constructed to disrupt MT-stimulated ATPase activity, inhibits HSET-dependent spindle elongation. The analogous mutations in Ncd and Kar3 uncouple MT-stimulated ATPase activity from MT sliding. We found that HSET-N593K also does not display MT-stimulated ATPase activity, but has a similar MT binding affinity to that of wild type HSET, showing that the failure to increase spindle length is not due to a non-functional protein, but rather is due to uncoupled ATPase activity. We also found that addition of a 5-fold excess of HSET-N593K to *Xenopus* CSF egg extracts did not stimulate bipolar spindle assembly compared to wild type HSET or XCTK2, suggesting that stimulation of spindle assembly requires sliding activity and that Kinesin-14 function is conserved among vertebrates. Whereas addition of a 10-fold excess of NLS-mutated XCTK2 to extracts results in an increase in spindle lengths, the addition of HSET-N593K decreases the length of bipolar spindles. Together these results support a model in which Ran-GTP in association with importin α/β regulate the action of XCTK2/HSET on MTs, and that cross-linking and sliding MTs is essential for XCTK2/HSET regulation of spindle assembly and morphology.

1906/B369

Spindle and MTOC Mechanisms for Kinesin-14 in Spindle Assembly and Function.

D. R. Simeonov, K. Kenny, J. Allen, E. Sadeghi, A. Moyer, J. L. Paluh; Biology, CBIS, Rensselaer Polytechnic Institute, Troy, NY

Segregation of sister chromatids into daughter cells for cell division occurs elegantly by formation of a bipolar spindle. The detailed mechanisms for spindle assembly are just being defined. This is in part because components are not well characterized and no single strategy

for assembly may exist. The presence or absence of centrosomes in animal cells necessitates both centrosome or microtubule prominent processes. A conserved theme in spindle assembly is microtubule interdigitation by Kinesin-5 kinesin-like proteins (Klps), countered by opposition from Kinesin-14 Klps. Genetic hints at the mechanisms of Kinesin-14 motors imply a different strategy than that of Kinesin-5. This lab is using comparative genomics along with site-directed mutagenesis, yeast two-hybrid and biochemical analysis and cell biology to define spindle assembly mechanisms. By analysis of alleles of microtubule organizing center (MTOC) proteins in fission yeast, Alp4, Alp6 and γ-tubulin, we determined that the MTOC can override opposition by Kinesin-14 in spindle assembly by altering microtubule organization and dynamic parameters. Two contributing sites on the surface of γ-tubulin were defined. The first site binds MTOC protein Alp4. The second adjacent domain is a kinesin binding site used by Kinesin-14 Pkl1 (Cell Cycle 7:1246-1253, 2008). This is the first definition of a tubulin binding site since discovery of Kinesin in 1985. To determine if features of Klp/γ-tubulin binding are shared by β-tubulin we generated chimeric tubulins with switched domains. In each case cells retain viability. Corresponding disabling mutations for Kinesin-14/γ-tubulin binding were also generated in β-tubulin. Their analysis will allow us to separate contributions of Kinesin-14/microtubule and Kinesin-14/MTOC mechanisms in spindle assembly. Finally we performed a comprehensive analysis of the Kinesin-14 Pkl1 stalk region to define critical domains as well as cross-species analysis of Kinesin-14 Klps Drosophila Ncd and Human HSET in fission yeast as full constructs and as Pkl1p chimeras. This work will define in detail the components in bipolarity mechanisms and refocuses importance on the MTOC for regulating microtubule parameters and spindle mechanisms.

1907/B370

Differential Effects of Kinesin-5 and Kinesin-8 Motors on Spindle Microtubule Plus-End Assembly in Budding Yeast.

M. Gardner[1], T. Salmon[2], K. Bloom[2], D. Odde[1]; [1]Department of Biomedical Engineering, University of Minnesota, Minneapolis, MN, [2]Biology, University of North Carolina, Chapel Hill, NC

During mitosis, sister chromatids congress to the spindle equator and are subsequently segregated via attachment to dynamic kinetochore microtubule (kMT) plus-ends. In vitro, the kinesin-8 molecular motor Kip3 is a processive plus-end directed motor that depolymerizes longer microtubules. Although Kip3 is present in the yeast nucleus and Kip3 deletion mutants have longer metaphase spindle lengths, Kip3 is not a major regulator of metaphase chromosome congression. Rather, we find that Cin8, the primary yeast kinesin-5 microtubule sliding motor, promotes depolymerization of longer kinetochore microtubules to mediate congression. Our analysis identified a model where kinesin-5 motors bind to kMTs, move to kMT plus ends, and upon arrival at a growing plus-end promote net kMT plus-end disassembly. Because deletion of either Kip3 or Cin8 results in similarly long astral microtubules outside of the mitotic spindle, we hypothesized that the organization of microtubules within the yeast mitotic spindle could contribute to differences in the interaction of Kip3 and Cin8 with spindle microtubules. Computer simulations that reproduce in vivo Cin8-GFP and Kip3-GFP localization and dynamics predict that the microtubule crosslinking properties of the tetrameric kinesin-5 motor Cin8 result in its concentration near to kMT plus-ends, while the non-crosslinking properties of the dimeric kinesin-8 molecular motor result in its concentration near to the plus-ends of the longer interpolar microtubules. Thus, the microtubule cross-linking properties naturally bias kinesin-5 motors towards kMT plus ends where they act to regulate kMT length, while kinesin-8 motors are biased toward interpolar microtubule plus ends where they act to regulate spindle length.

1908/B371

Kinesin-5-Dependent Poleward Flux and Spindle Length Control in Drosophila Embryo and Neuroblast Mitosis.

I. Brust-Mascher, P. Sommi, D. Cheerambathur, J. Scholey; Mol and Cell Biol, Univ of California - Davis, Davis, CA

We tested the hypothesis that the persistent sliding apart of interpolar (ip) microtubules (MT) by the homotetrameric kinesin-5, KLP61F, controls poleward flux and spindle length in Drosophila, using antibody microinjection, gene dosage experiments and klp61f mutants to dissect the different roles of this mitotic motor. In wild type and Ncd null mutant embryos anti-KLP61F produced a spatial gradient, visible in GFP-KLP61F transgenic embryos, in which spindles proximal to the injection site contained no detectable KLP61F but those at more distal sites contained correspondingly more motor. The resulting mitotic defects exhibited a gradient, in which spindles with no detectable KLP61F collapsed at prometaphase, while other spindles had defects, that became less severe with increasing amounts of motor. Gene dosage experiments in embryos and time-lapse microscopy of living klp61f mutant embryos and larval brains are consistent with the antibody injection experiments. The observed defects reveal that, following nuclear envelope breakdown, KLP61F drives persistent microtubule bundling and sliding. In contrast to previous results, we find that this outward sliding of antiparallel microtubules by kinesin-5 contributes to the poleward flux of both ipMTs and kinetochore MTs, and to the length of the metaphase spindle. KLP61F activity does not appear to contribute to chromosome congression, but it does contribute to the rate of anaphase A. In addition KLP61F driven MT sliding maintains the prometaphase spindle by antagonizing Ncd and an additional unknown force-generator, and drives anaphase B. However, while KLP61F is vital for anaphase B, the rate of spindle elongation is relatively insensitive to the motor's concentration, as predicted by models for anaphase B. In summary, our results show that the kinesin-5 KLP61F deploys its MT-MT crosslinking and antiparallel MT sliding activity to perform multiple functions in Drosophila mitosis.

1909/B372

A Mitotic Function of Epsin1 Involving the Membrane-Bending ENTH Domain.

Z. Liu, Y. Zheng; Department of Embryology, Carnegie Institution of Washington, Baltimore, MD

How various membrane compartments are organized in mitosis for their partitioning into daughter cells and whether proper membrane organization influences mitotic spindle morphogenesis are unclear. Although studies have shown that several proteins with well-established functions in interphase membrane trafficking also regulate various aspects of mitosis, whether these proteins could influence spindle morphogenesis by regulating mitotic membrane organization is unknown. We report that the endocytic accessory protein Epsin1 facilitates mitotic spindle organization in tissue culture cells and in Xenopus egg extracts. Using Epsin1 that harbors a point mutation in the ENTH domain, we show that the ability of Epsin1 to induce membrane curvature is required for proper spindle morphogenesis. Since the membrane bending function of Epsin1 facilitates spindle assembly in cell free Xenopus egg extracts, our studies reveal that Epsin1 modulates mitotic membrane morphology to facilitate spindle assembly, which is independent of Epsin's role in endocytosis.

1910/B373

Adenomatous Polyposis Coli (APC) Mutants Impair the Mitotic Checkpoint through Direct Interaction with Mad2.

J. Zhang; Department of Pathology and Cell Biology, Columbia University College of Physicians and Surgeons, New York, NY

The majority of colorectal tumors are aneuploid due to underlying chromosome instability (CIN) phenotype, where a defective mitotic checkpoint is implicated. Adenomatous polyposis coli (APC), a tumor suppressor gene that commonly mutated in colon cancers, has been suggested in causing CIN; however, the molecular mechanism remains unresolved. In this study, we found that addition of such an N-APC mutant in Xenopus egg extracts inactivates the mitotic checkpoint. Expressing a tumor-associated N-APC mutant in mammalian cultured cells with an intact mitotic checkpoint produces anaphase onset with unaligned chromosomes.More importantly,we demonstrated an interaction of tumor-associated APC N-terminal truncated mutants (N-APC) with Mad2, an essential mitotic checkpoint protein, providing a direct molecular support for linking the tumor-associated APC mutations to the generation of CIN in human cells. N-APC interacts with Mad2 in Xenopus egg extracts, cultured colon cancer cells, and in vitro with purified recombinant proteins. Interaction between N-APC and Mad2 decreases the soluble pool of Mad2, which is essential for Mad2 cycling and releasing from unattached kinetochores to produce a diffusible "wait anaphase" signal. These will be important for understanding the generation of CIN, a possible driving force of tumorigenesis, in colon cancer progression.

1911/B374

The Chromatin Remodeling Factor Rsf-1/HBxAP Mediates the HBx/hBubR1 Interaction.

S. Chae[1,2], S. Kwon[1], J. Lim[3], C. Lee[4], D. Kang[3], H. Cho[1,2]; [1]Department of Biochemistry and Molecular Biology, Ajou University School of Medicine, Suwon, South Korea, [2]Department of Molecular and Science Technology, Ajou University, Suwon, South Korea, [3]Department of Life Science, Ewha Womans University, Seoul, South Korea, [4]Department of Molecular Cell Biology, Sungkyunkwan University, Suwon, South Korea

Hepatitis B virus X (HBx), encoded by HBV genome, deeply involves in the development of HBV-mediated liver cancer. We have recently reported that HBx binds hBubR1, a major component of the mitotic checkpoint (Oncogene. 27(24):3457-64, 2008). Here, we precisely map the region of HBx that interacts with hBubR1 by in vivo co-immunoprecipitation assay. The full-length of HBx1-154 as well as the HBx51-154 retaining two Kunitz domains strongly bind hBubR1 whereas deletion of them profoundly reduced the hBubR1 binding. Meanwhile, hBubR1 region comprising of a.a 476-687 was found to be essential in HBx binding. Surprisingly, in vitro binding assay revealed a notably weak HBx/hBubR1 binding, suggesting that a strong HBx/hBubR1 interaction in vivo require other proteins. We found that the HBx/hBubR1 interaction in vivo was abolished by siRNA against Rsf-1/HBxAP, known as a chromatin remodeling factor, but not by HBxIP siRNA and depletion of Rsf-1/HBxAP partially diminished the HBx-mediated mitotic aberrations. Together, our data show that the Rsf-1/HBxAP mediates the HBx/hBubR1 interaction in vivo. This provides a novel mechanism for mitotic aberrations by a viral pathogen, which contributes to the accumulation of genomic instability in hepatocarcinogenesis.

1912/B375

Mutual Dependence of Mob1 and CPC for Localization during Mitosis.

L. Wilmeth, S. Shrestha, C. Shuster; Biology, New Mexico State University, Las Cruces, NM

The timely coordination of chromosome segregation and cytokinesis is essential to ensure that each daughter cell receives the correct complement of chromosomal and cytoplasmic material. In yeast, the exit from mitosis and the initiation of cytokinesis is coordinated by a signaling cascade whose terminal components include an NDR family protein kinase and a non-catalytic regulatory subunit (Mob1). There are five mammalian Mob1 genes, all of whom display redundant localization patterns, localizing at the centrosomes and kinetochores in early mitosis, then to the spindle midzone at the onset of cytokinesis. The localization dynamics of Mob1 was highly reminiscent of both Polo-like kinase (Plk1) and the chromosomal passenger complex (CPC), two key cell cycle regulators linked genetically to Mob1 in yeast. However, while Plk1 depletion resulted in a loss of Mob1 from spindle poles, it failed to affect Mob1 recruitment to kinetochores. Disruption of CPC signaling by siRNA depletion or chemical inhibition resulted in a loss of Mob1 from both kinetochores and the spindle midzone without disrupting centrosomes recruitment. Mob1 and the CPC appeared to be mutually dependent on each other for chromosomal localization, since Mob1A depletion altered Aurora B localization at centromeres during prometaphase from a broad pan-centromeric staining pattern to a more streaked, microtubule-associated appearance. This altered recruitment/retention of Aurora B appeared to have functional consequences, as evidenced by the increase in prometaphase-like mitotic figures suggestive of congression defects. Together, these results suggest that Mob1 and the chromosomal passenger complex are mutually dependent on each other for localization early in mitosis, and that Mob1 may be playing a novel role in regulating mitotic progression at the kinetochores through its interaction with the CPC.

1913/B376

Polo-Like Kinase-1-Mediated Regulation of Slingshot-1 by Phosphorylation.

M. Otsuki, C. Ikebe, K. Ohashi, K. Mizuno; Graduate School of Life Sciences, Tohoku University, Sendai, Japan

Cofilin has F-actin severing and depolymerization activities, and plays a principal role in mitosis and cytokinesis in animal cells. Cofilin activity is negatively regulated by phosphorylation at Ser-3 by LIM-kinase-1 (LIMK1) and reactivated by a protein phosphatase Slingshot-1 (SSH1). We previously reported that the level of the Ser-3-phosphorylated cofilin (P-cofilin) periodically changes during the cell division cycle in HeLa cells; it increases in prometaphase and metaphase, then is gradually dephosphorylated in telophase and cytokinesis. Cell cycle-associated changes in LIMK1 and SSH1 activities correlate well with the changes in the level of P-cofilin. Whereas the change in phosphatase activity of SSH1 is thought to be important for cell cycle-dependent control of cofilin activity, responsible kinases or phosphatases that regulate SSH1 activity during the cell cycle have remained to be elucidated. By yeast two-hybrid screening, we identified Polo-like kinase (Plk)-1, a serine/threonine kinase, as one of the SSH1-binding proteins. Plk1 was found to specifically interact with SSH1 through its Polo-box domain and Ser-689 within the carboxy-terminal region of SSH1, using co-immunoprecipitation assay. Plk1 phosphorylated SSH1(WT), but neither SSH1(S689A) mutant nor dephosphorylated SSH1(WT), indicating that the priming phosphorylation at Ser-689 is required for Plk1-mediated SSH1 phosphorylation. Using a series of deletion and point mutants of SSH1, we identified some of

the Plk1-catalyzed phosphorylation sites in the amino-terminal region of SSH1. We are currently examining the effect of these phosphorylations on the activity of SSH-1 and its phenotypic outcomes in cultured cells. As Plk1 is known to be an important cell cycle regulator, a model of how Plk1 modulates SSH1 activity during the cell cycle will be discussed.

1914/B377

Genome Stability Is Ensured by Temporal Control of Kinetochore-Microtubule Attachment.

S. Bakhoum[1,2], S. Thompson[1,2], D. Compton[1,2]; [1]Biochemistry, Dartmouth Medical School, Hanover, NH, [2]Norris-Cotton Cancer Center, Lebanon, NH

Most solid tumors are aneuploid and many frequently mis-segregate chromosomes. This chromosomal instability is commonly caused by persistent maloriented attachment of chromosomes to spindle microtubules. Kinetochore microtubule attachments must be adequately stable to allow for timely inactivation of the spindle checkpoint and chromosome segregation, yet these attachments must also be sufficiently dynamic to permit correction of malorientations. How this balance is achieved is unknown, and the permissible boundaries of attachment dynamics essential for genome stability remain poorly understood. Here we show that two microtubule-depolymerizing kinesins, Kif2b and MCAK, stimulate kinetochore-microtubule dynamics during distinct phases of mitosis to correct malorientations. Few-fold reductions in kinetochore-microtubule turnover, particularly in early mitosis, induce severe chromosome segregation defects. In addition, we show that stimulation of microtubule dynamics at kinetochores restores chromosome stability to chromosomally unstable tumor cell lines, establishing a causal relationship between deregulation of kinetochore-microtubule dynamics and chromosomal instability. However, further two-fold increase in turnover rates during metaphase leads to persistent spindle checkpoint activation and mitotic arrest. Thus, precise temporal control of microtubule attachment to chromosomes during mitosis is central to genome stability and proper mitotic progression in human cells.

1915/B378

In Vitro Analysis of Mitotic APCCdc20 Inhibition by Budding Yeast Mad2, Mad3 and Bub3.

S. C. Schuyler[1], N. A. Larsen[2], S. C. Harrison[2], A. Murray[1]; [1]MCB, Harvard University, Cambridge, MA, [2]Jack Eileen Connors Structural Biology Laboratory, HHMI, Harvard Medical School, Boston, MA

The mitotic spindle checkpoint is a biochemical pathway that monitors microtubule attachment and tension at the kinetochores. Activation of this pathway leads to the inhibition of the Cyclosome or Anaphase-Promoting Complex (APC), an E3 ubiquitin ligase that is required for the metaphase-anaphase transition. One mechanism by which the checkpoint is thought to inhibit APC activity is by the formation of the Mitotic Checkpoint Complex (MCC). This complex contains the APC co-factor Cdc20, as well as three spindle checkpoint proteins, Mad2, Mad3 and Bub3. The goal of this study is to explore the mechanism of inhibition of APC^{Cdc20} by Mad2, Mad3 and Bub3 by employing an *in vitro* mitotic APC^{Cdc20} assay from budding yeast. Using pure recombinant checkpoint proteins we have measured the relative contribution of each component of the MCC to APC^{Cdc20} inhibition. The EC50, the concentration giving 50% of a checkpoint protein's maximal inhibition, was measured using titrations. We observe that Bub3 alone is a weak inhibitor, with an EC50 of about 10 µM. We also observe that Bub3 strengthens Mad3 inhibition. The EC50 for Mad3 alone is 250 nM, but the EC50 of the Mad3-Bub3 complex is 85 nM. The Hill slopes for the Mad3, and the Mad3-Bub3 complex titrations are >1. Furthermore, in the presence of a high amount of Mad3-Bub3 complex the small amount of substrate that is ubiquitinylated is only modestly modified as poly-ubiquitinylation appears to be suppressed. In contrast, the EC50 for Mad2 is 2.0 µM, and in the presence of high levels of Mad2 the small amount of substrate that is ubiquitinylated appears to be fully poly-ubiquitinylated. Finally, we observe synergism between Mad2 and Mad3-Bub3 inhibition. We conclude that each checkpoint protein contributes to a unique aspect of MCC function that gives rise to the full inhibition of APC^{Cdc20}.

1916/B379

Poleward Transport of TPX2 in Mammalian Mitotic Spindles.

N. Ma[1], N. Ferenz[1], C. Fagerstrom[1], U. S. Tulu[3], A. Wilde[2], P. Wadsworth[1]; [1]Biology, University of Massachusetts, Amherst, MA, [2]Molecular Genetics, University of Toronto, Toronto, ON, Canada, [3]Biology, Duke University, Durham, NC

Bipolar spindle assembly is accomplished by the combined activity of dynamic microtubules, mitotic motors and microtubule binding proteins. One such binding protein, TPX2, is a Ran-regulated spindle assembly factor required for kinetochore fiber formation. As a first step in understanding the dynamic properties of TPX2 and how it mediates kinetochore fiber formation, we generated an LLC-Pk1 cell line expressing PA-EGFP-TPX2. Cells expressing the construct progressed normally through mitosis and the distribution of the exogenous protein was indistinguishable from endogenous TPX2. Photoactivation experiments revealed that PA-EGFP-TPX2 moves poleward in prometaphase and metaphase, at rates (1.57 + .87 and 0.84 + .44 um/min, respectively) similar to poleward flux in metaphase LLC-Pk1 cells (1.37 + .47 um/min; Ferenz and Wadsworth, 2007). In anaphase, however, PA-EGFP-TPX2 on interzonal microtubules is static, demonstrating that TPX2 dynamics is regulated throughout mitosis. To assess the contribution of microtubule flux to the dynamics of TPX2, we inhibited either Eg5 or Kif2a, motors previously shown to contribute to flux. Inhibition of each motor reduced the rate of TPX2 poleward motion at prometaphase. Because TPX2 was identified as a factor required for dynein-dependent targeting of the kinesin Xklp2 to spindle poles, we also examined the contribution of dynein to TPX2 dynamics. Following inhibition of dynein/dynactin with p150-CC1, TPX2 motion was greatly reduced and TPX2 distribution was shifted toward microtubule plus-ends. These results demonstrate that the movement of TPX2 depends on its location in the spindle, the stage of mitosis, and, either directly or indirectly, the activity of dynein, Eg5 and Kif2a. We hypothesize that TPX2 clusters parallel microtubules in the kinetochore fiber and near the pole and that its dynamic distribution reflects the net, local activity of molecular motors.

1917/B380

The Microtubule-Associated Protein Stu1p Plays a Phosphorylation-Dependent Role in Anaphase Spindle Dynamics in Saccharomyces Cerevisiae.

A. E. Faust[1], C. C. Wong[2], J. R. Yates[2], D. Drubin[1], G. Barnes[1]; [1]Molecular and Cell Biology, UC Berkeley, Berkeley, CA, [2]Scripps Research Institute, La Jolla, CA

Cells depend on chromosome segregation for survival, and for all eukaryotic cells, proper chromosome segregation depends on the establishment and maintenance of a microtubule-based mitotic spindle. In the budding yeast Saccharomyces cerevisiae, the integrity of the mitotic spindle is maintained by a number of microtubule-associated proteins (MAPs), including Ase1p, Slk19p, and Stu1p. The purpose of this study is to understand the role of the essential MAP Stu1p in spindle stabilization and dynamics. Previous studies have identified STU1 as an essential gene, and Stu1p as a critical structural component of the mitotic spindle during metaphase and anaphase. A putative member of the CLASP/Mast/Orbit family of plus-end tracking proteins, Stu1p has been shown to bind microtubules in vitro and localize to the kinetochore and spindle midzone. However, its role in spindle stabilization and maintenance, as well as its regulation throughout the cell cycle, is not known. Through phosphopeptide enrichment and mass spectrometric analysis, we found that Stu1p is phosphorylated in vivo at a Cdc28p (CDK1) target consensus site. Using targeted mutagenesis and live cell microscopy, we found that both the phosphomutant and phosphomimic Stu1p mutants cause defects in the transition between the fast and slow phases of spindle elongation during anaphase B. Previous work has shown that the MAP Ase1p is also dependent on Cdc28p phosphorylation for proper function in mitotic spindle integrity, so MAP regulation by Cdc28p may be a fundamental way for cells to regulate spindle dynamics. Through mass spectrometry of Stu1p purified from yeast extracts, we found that Stu1p physically interacts in vivo with Slk19p, a MAP that functionally interacts with Ase1p and has recently been shown to play a role in the transition between fast and slow phase of spindle elongation during anaphase B. Attempts to identify additional Stu1p-interacting proteins through yeast 2-hybrid assays and efforts to identify functional domains of Stu1p are ongoing. These results indicate that Stu1p plays a critical phosphorylation-dependent role in maintaining the yeast mitotic spindle during anaphase.

1918/B381

Role of PAK-PIX Signaling to Cofilin in Mitosis.

L. Tahtamouni[2,1], J. DeLuca[1], J. Bamburg[1], O. Wiggan[1]; [1]Department of Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, [2]Department of Biology, Hashemite University, Zarqa, Jordan

The p21-activating kinases (PAKs) are downstream effectors of Rac and Cdc42. PAKs are targeted and activated at different cellular sties through interaction with the Rho GEF PIX. PAKs regulate the actin cytoskeleton in part through activating LIM kinase (LIMK), which in turn inactivates cofilin, an actin-dynamizing protein. This regulatory cascade has been implicated in the regulation of various aspects of cell division. Here we show that disruption of the PAK-PIX interaction through expression of a peptide comprising the PIX binding motif of PAK1 (PIX-BD) results in distinct cell division defects. PIX-BD expression results in cell multinucleation, and in altered spindle orientatation. PIX, phospho-PAK, phospho- LIMK1/2 (pLIMK)and phospho-cofilin localize to distinct mitotic structures such as the centrosome and cleavage furrow in multiple cell types. PIX-BD expression in HeLa cells results in altered pLIMK levels at these structures suggesting a role for the LIMK/cofilin pathway downstream of PAK-PIX signaling during mitosis. Our on-going studies will define the mechanisms by which PAK-PIX signaling through LIMK/cofilin regulate normal cell division. Supported in part by NIH grants NS43115(OW,JRB) and NS40371(JRB)

1919/B382

Mutual Regulation of Cyclin Dependent Kinase and the Mitotic Exit Network.

C. König, H. Maekawa, E. Schiebel; Zentrum für Molekulare Biologie Heidelberg (ZMBH), DKFZ-ZMBH Allianz, Heidelberg, Germany

Mitotic exit defines the transition from mitosis into G1 phase of the cell cycle. In all eukaryotic cells mitotic exit is driven by a decrease in the activity of mitotic cyclin dependent kinase (Cdk1 or Cdc28 in budding yeast) even though the mode and kinetics of its inactivation varies between organisms. The mitotic exit network (MEN) is a centrosome-associated, GTPase driven signalling cascade that regulates the localization of the conserved phosphatase Cdc14 and thereby controls mitotic exit. Here, we show by live cell imaging that in early anaphase Cdc28 becomes recruited to the yeast centrosome, the spindle pole body (SPB), in dependence of the MEN. Cdc28 binds preferentially to the SPB in the mother cell body that does not carry the Bub2-Bfa1 GAP complex, which is an inhibitor of the MEN and of Cdc28 binding to the SPB in the daughter cell. With biochemical and cell biological approaches we found that Mob1 is a substrate of cyclin dependent kinase and becomes dephosphorylated with mitotic exit by Cdc14. Genetic and biochemical data suggest that Cdc28 regulates the MEN in two ways. First, Cdc28 phosphorylation of Cdc15 and Mob1 inhibits a rearrangement of the MEN in mid-anaphase. Second, phosphorylation of Dbf2-Mob1 reduces the specific activity of Mob1-Dbf2 kinase. Together, these data revise our understanding on the spatial regulation of the MEN during the cell cycle. Whereas the MEN in the daughter is inhibited by the Bub2-Bfa1 complex, the MEN in the mother cell body is regulated by Cdc28.

Cytokinesis I (1920 – 1944)

1920/B383

mid1p Associates with the Mitotic Kinase cdr2p to Position the Division Plane in S. Pombe.

M. Almonacid[1], J. Moseley[2], J. Janvore[1], A. Mayeux[1], P. Nurse[2], A. Paoletti[1]; [1]Institut Curie/CNRS UMR144, Paris, France, [2]Rockefeller University, New York, NY

Cells must position their division plane properly to ensure accurate segregation of chromosomes and organelles. In S. pombe, the anillin-like protein mid1p defines the division plane and recruits contractile ring components to the medial cortex at mitosis. How mid1p is targeted to the medial cortex remains unclear. mid1p contains a C-terminal amphipathic helix that facilitates association with the plasma membrane, but mutations disrupting this helix do not dramatically affect mid1p function or division plane placement. Here, we show that the N-terminal domain of mid1p is anchored to the medial cortex by the mitotic kinase cdr2p. mid1p and cdr2p physically associate, and we generated mutations that disrupt this interaction. Similar to cdr2 deletion, these mutations impair mid1p cortical anchoring during interphase but have little impact on division plane positioning. However, cdr2p becomes essential for division plane definition when C-terminal anchoring of mid1p is abolished or when mid1p nuclear shuttling is perturbed. This synergistic relationship suggests that mid1p-cdr2p interactions functionally overlap with the mid1p C-terminal helix to establish medial placement of the division plane. We propose that interphase signals

generating cdr2p localization to the medial cortex act redundantly with a mid1p nuclear shuttling mechanism to promote proper division plane specification.

1921/B384

Quantitative Studies of Anillin in Cytokinesis.

L. Zhang, T. Nguyen, J. F. Dorn, A. S. Maddox; Institute for Research in Immunology and Cancer, Université de Montréal, Montréal, QC, Canada

Anillin is a contractile ring protein that binds actin filaments, myosin, and the septins via distinct domains, and is therefore thought of as a cytoskeletal crosslinker. While conserved across phylogeny, Anillins play varied roles in cytokinesis. The first mitotic cytokinesis in the *C. elegans* embryo does not require Anillin (ANI-1), but the geometry of furrow closure is altered from asymmetric to symmetric following Anillin depletion. Symmetric furrowing is accompanied by increased sensitivity to inhibition of contractility, suggesting that Anillin helps to make cytokinesis robust. Interestingly, Anillin is absolutely required for proper polar body extrusion, the specialized cytokinesis that terminates maternal meiosis. The topology of cleaving a polar body from the flat oocyte surface seems especially challenging. Anillin's requirement in this event allows us to inspect its essential functions when redundant mechanisms do not compensate. Preliminarily, we find that Anillin is required for contractile ring organization and cortical flexibility in meiotic cytokinesis. We hypothesize that Anillin amplifies symmetry breaking in the mitotic contractile ring by virtue of its ability to crosslink multiple filament-forming proteins (actin, myosin and the septins). Using transgenic *C. elegans* strains expressing GFP-tagged constructs, we are testing whether Anillin truncations lacking one more filament-interaction domain can replace endogenous Anillin function following its depletion by RNAi. Both full-length Anillin and a truncation lacking the predicted septin-binding domain rescued the embryonic lethality following depletion of endogenous Anillin. In these cells, the speed and success of furrowing were quantitatively normal in the presence, or following depletion, of endogenous Anillin. Full-length GFP-Anillin conferred asymmetric localization and furrowing. However, when endogenous Anillin was depleted from cells expressing Anillin lacking the predicted septin-binding domain, the contractile ring closed symmetrically. Thus, Anillin's interaction with septins appears to be required for symmetry breaking. We are continuing these analyses with truncations lacking other filament-interaction domains.

1922/B385

Embryonic Cells Accurately Position the Cytokinetic Furrow Even When No Microtubules Approach the Cortex.

G. Von Dassow[1], K. Verbrugghe[3], A. Miller[3], J. Sider[2,1], W. Bement[3,1]; [1]Friday Harbor Labs, University of Washington, Friday Harbor, WA, [2]Amundsen High School, Chicago, IL, [3]Department of Zoology, University of Wisconsin, Madison, WI

Nearly all models of animal cytokinesis invoke hypothetical delivery of signals to the cortex by microtubules of the mitotic apparatus; most of the debate has focused on whether astral microtubules inhibit or stimulate contractility, or whether microtubules of the asters or central spindle convey the key positional information. Using a new method to image microtubules in live urchin embryos, we investigated the spatial relationship between arrival of microtubule ends at the cortex and onset of furrowing or Rho activation. In 8-cell and older embryos, astral microtubules reach the cortex everywhere well before furrowing, but more densely outside the equator. Recent studies in urchin zygotes and cultured cells show that furrows are associated with stable microtubules. However, we find that in later embryos, astral microtubules are kinematically stable everywhere after mid-anaphase, and there is no spatial orientation to the dynamically-stable subset of astral microtubules. Next we treated urchin embryonic cells with Trichostatin A to abolish astral microtubules without interfering with spindle function. Although these cells completely lack detectable microtubules connecting any part of the mitotic apparatus with any portion of the cell surface, they nevertheless accurately position the cleavage furrow. In contrast, we show using enucleated cytoplasts that simple juxtaposition of two asters is inadequate to induce furrowing; asters must be sufficiently far apart to do so. Asters are thus neither required nor, strictly, sufficient for furrow induction. Even so, partial ablation of one or both centrosomes shows that either the aster or the centrosome itself somehow inhibits furrowing. The simplest interpretation is that the mitotic apparatus emits a diffusible cue and that the microtubule array either refines the distribution of this cue or amplifies it locally.

1923/B386

During Cytokinesis, the Spindle Midzone Dictates Furrow Trajectory and the Separated Asters Suppress the Formation of Ectopic Furrows.

L. Lewellyn, A. B. Desai, K. Oegema; Cellular and Molecular Medicine, Ludwig Institute for Cancer Research, La Jolla, CA

During cytokinesis, furrow formation is directed by signals from the centrosomal asters and spindle midzone. To study the coordination of these signals, we analyze the consequences of inhibiting aster separation during the first embryonic division in *C. elegans*. Depletion of the spindle protein TPXL-1 shortens the metaphase spindle, increasing the time required for the centrosomes to achieve a normal extent of separation following anaphase onset. In TPXL-1 depleted embryos, furrow formation is delayed until the asters approach a normal extent of separation. When centrosome separation is prevented by simultaneous inhibition of Gα signaling and TPXL-1, the spindle midzone drives the formation of multiple furrows at different points spanning the central third of the embryo that simultaneously ingress towards the spindle center. Thus, although the midzone can dictate the trajectory of ingressing furrows, the cell requires input from the separated asters to limit ingression to a single furrow.

1924/B387

Structural Memory in the Contractile Ring makes Cytokinesis Timing Independent of Initial Cell Size.

A. Carvalho, A. Desai, K. Oegema; Department of Cellular and Molecular Medicine, University of California-San Diego, Ludwig Institute for Cancer Research, La Jolla, CA

Animal cell cytokinesis is accomplished by constriction of a cortical contractile ring. Here we explore the inherent properties of the contractile ring by investigating how the rate of ring closure scales with respect to initial ring size. We show that during the first five embryonic divisions in *C. elegans*, contractile ring constriction occurs in two phases - an initial phase at a constant rate followed by a second phase at an exponentially decreasing rate. For all divisions, the transition between the two phases occurs at a fixed perimeter and requires an

intact spindle midzone. Remarkably, despite the wide range of initial perimeters, constriction completes in the same time for all five divisions. This is explained by a strict proportionality between the constant rate of constriction and initial size—contractile rings that are initially larger constrict at a proportionally higher constant rate. Characterization of contractile ring components revealed that the total amount of myosin II, anillin and the septins, decreases in proportion to ring perimeter during constriction. In the case of myosin II, this occurred in the absence of rapid exchange with the cytoplasmic or cortical pools. These results indicate that the contractile ring progressively disassembles while retaining a structural memory that renders constriction time independent of its initial size. We propose that the contractile ring is built from units that are of fixed size and shorten at a fixed rate - larger contractile rings contain more units than smaller rings and constrict at a proportionally faster rate. Structural memory, which makes cytokinesis scalable across a wide range of initial sizes, is likely to be a highly conserved property of the contractile ring that explains how mitotic exit and cytokinesis are coordinated when cell size, and hence the distance the contractile ring must traverse, varies during embryogenesis and in other biological contexts.

1925/B388

RSK-1 Depletion Induces Cytokinesis Defect through Decrease of Anillin Expression.

H. Nam[1,2], S. Jang[1,2], K. Hong[3], C. Bae[3], J. Lee[1,2]; [1]Department of Biochemistry and Molecular Biology, Ajou University School of Medicine, Suwon, South Korea, [2]Department of Molecular Science and Technology, Graduate School of Ajou University, Suwon, South Korea, [3]Department of Molecular Cell Biology and Samsung Biomedical Research Institute, Sungkyunkwan University School of Medicine, Suwon, South Korea

The mitogen-activated protein (MAP) kinase pathway regulates various cellular processes, including gene expression, cell proliferation, and cell cycle. Here, we have examined the potential involvement of p90 ribosomal S6 kinase (RSK) as a key player in the regulation of mitosis. To assess the role of RSK during the cell cycle, siRNAs targeting each of the two isoforms (RSK-1, 2) were transfected into unsynchronized HeLa cells. Interestingly, depletion of RSK-1, but not RSK-2, resulted in an accumulation of binucleated cells compared with control cells. To examine whether the formation of binucleated cells in RSK-1 depleted cells was induced as a result of cytokinesis defect, cell division was monitored by time-lapse imaging. Unlike control or RSK-2 depleted cells, RSK-1 depleted cells failed to complete ingression or abscission of cytokineis, resulting in the formation of binucleated cells. These results suggest that RSK-1 is required for completion of cleavage furrow ingression or abscission during cytokinesis. To figure out whether there were cytokinesis regulators controlled by RSK-1 depletion, we undertook a microarray analysis. Among genes changed by RSK-1 depletion, expression of anillin, a putative scaffolding protein that can bind F-actin, myosin II, and RhoA, decreased in both mRNA and protein levels. In addition, we observed that localization of anillin at cleavage furrow was reduced by RSK-1 depletion, which was followed by the decrease of RhoA localization at cleavage furrow. These results strongly suggest that decrease of expression and localization at cleavage furrow of anillin by RSK-1 depletion induces cytokinesis defect.

1926/B389

Anillin Stabilizes Active RhoA in Cytokinesis.

A. Piekny[1], M. Glotzer[2]; [1]Biology, Concordia University, Montreal, QC, Canada, [2]Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL

Anillin is a conserved actin and myosin binding protein that is an early marker for the location of the contractile ring in eukaryotes from yeast to human cells. Anillin co-localizes with RhoA and contains a highly conserved and essential region in the C- terminus (AHD or anillin homology domain) that directly interacts with RhoA. When anillin is depleted from human cells the active pool of RhoA, visualized by trichloroacetic acid (TCA) fixation, is lost from the site of the furrow. Anillin depletion causes a unique phenotype, where furrows partially ingress followed by repeated oscillations and furrow regression. This phenotype likely arises due to the uncoupling of active myosin from the equatorial plane of the cell. The inability to maintain myosin in the centre of the cell, coupled with the decrease in TCA-stabilized RhoA supports a feed back role for anillin in the production and/or maintenance of active RhoA at the furrow. Further characterization of anillin-depleted cells revealed that a fraction of the cells exhibited a phenotype that resembled weak depletion of the upstream activator for RhoA, the RhoGEF Ect2, consistent with a reduction in the levels of active RhoA. We are currently investigating the molecular mechanisms through which anillin positively regulates RhoA activity.

1927/B390

Evaluating the Chromosomal Passenger Complex and MKLP1 in Directing the Final Events in Cell Division in Sea Urchin Embryos.

H. Argiros, C. J. Holguin, C. Shuster; New Mexico State University, Las Cruces, NM

Cleavage plane determination has been an area of investigation for over a century, and classic micromanipulation experiments have demonstrated that overlapping astral arrays are sufficient for furrow specification in echinoderm eggs. Other models have implicated the chromosomal passenger (CPC)- and centralspindlin complexes to organize the spindle midzone, locally activate Rho-GTPase, and assemble the contractile ring. Since experiments performed in echinoderm eggs have demonstrated that the cleavage plane may be established in the absence of a central spindle and chromosomes, we have focused on evaluating the CPC and MKLP1 during cytokinesis in sea urchin embryos. Inhibition of Aurora B activity resulted in furrow regression, although astral microtubule elongation and furrow initiation was normal. There was, however, a lack of organized microtubule arrays associated with the ingressing furrow. Both MKLP1 and the CPC component Survivin initially localized to the former metaphase plate upon anaphase onset, and spread outward towards the cortex as anaphase progressed, localizing to a narrow region of the cell equator. MKLP1 localized to furrow microtubule arrays during cytokinesis, and microtubule bundling was lost in the absence of Aurora B activity. To examine whether MKLP1 and the CPC localized to cleavage planes lacking a spindle midzone, binucleate cells were generated by reversibly blocking the first cleavage, and probed for survivin and MKLP1 during the second division. Both MKLP-1 and survivin localized to the primary axis of cleavage (in the plane of the preceding metaphase plate), but were absent from the secondary axis, where furrows are generated from astral microtubules emanating from unrelated spindle poles. Together, these results suggest that in cells where astral signaling specifies the cleavage plane, there are two partially redundant

signaling mechanisms for cytokinesis, and that CPC-centralspindlin signaling acts as the secondary signal required for the maintenance and completion of contractile ring ingression.

1928/B391

Negative Regulation of Rac and Arp2/3 by CYK-4 Is Essential for Cytokinesis.

J. C. Canman[1,2], L. Lewellyn[1], S. J. Smerdon[3], A. B. Desai[1], B. Bowerman[2], K. Oegema[1]; [1]Dept of Cell & Mol Medicine, Ludwig Institute for Cancer Research, La Jolla, CA, [2]Institute of Molecular Biology, University of Oregon, Eugene, OR, [3]National Institute for Medical Research, London, United Kingdom

Cytokinesis is the physical division of one cell into two. In animal cells, cytokinesis is driven by RhoA-dependent constriction of a contractile ring enriched in actin and myosin II, which forms around the cell equator. To ensure that each daughter cell receives a single genomic complement, contractile ring assembly is directed by the anaphase spindle. In particular, a set of microtubule bundles that forms between the separated chromosomes called the "central spindle" is thought to signal to the cortex to promote contractile ring formation. Assembly of the central spindle requires Centralspindlin, a conserved hetero-tetrameric complex consisting of two molecules of the kinesin ZEN-4 (MKLP1/CHO1/Pavorotti) and two molecules of CYK-4 (MgcRacGAP/RacGAP50C), a protein which contains a GAP domain for Rho family GTPases. The importance of the CYK-4 GAP domain and the identity of the Rho family GTPase that it targets have been unclear. Here, we identify two separation-of-function mutations which selectively affect the GAP domain of CYK-4 without compromising its ability to interact with ZEN-4 or promote central spindle assembly. The GAP domain mutations result in a cytokinesis defect that mimics the Centralspindlin loss of function phenotype- the contractile ring forms and partially constricts, but then regresses. Remarkably, inhibition of Rac or its downstream effector Arp2 specifically rescues the cytokinesis defects observed in the GAP mutants. These results indicate that the essential function of Centralspindlin in cytokinesis is to locally inactivate Rac to prevent Arp2/3 complex activation.

1929/B392

Actomyosin-Ring-Independent Roles of Rho-Family GTPases during Cytokinesis in Saccharomyces Cerevisiae.

M. Onishi, N. Ko, R. Nishihama, J. Pringle; Department of Genetics, Stanford University School of Medicine, Stanford, CA

During cytokinesis, the plasma membrane invaginates into the cytosol to form the cleavage furrow. It is widely believed that Rho-GTPase-mediated formation of the actomyosin ring (AMR) and its contraction are essential for this ingression. However, in some cell types, cytokinesis can be accomplished even in the absence of the AMR. In the budding yeast *S. cerevisiae*, the cleavage furrow still ingresses in AMR-deficient myosin II-null cells, apparently driven by sequential deposition of two types of extra-cellular matrix, the primary septum (PS; chitinous cell wall within the cleavage furrow) and the secondary septum (SS; glucan-rich cell wall sandwiching the PS). We and others have previously identified several proteins playing pivotal roles in membrane invagination and PS formation, including Cyk3p and Hof1p. To understand the molecular roles of these proteins, we performed multi-copy-suppressor screens using *cyk3Δ hof1Δ* and *cyk3Δ myo1Δ* double mutants, both of which are nearly inviable due to their deficiency in PS formation. Many of the genes identified are related to two small GTPases, Rho1p and Cdc42p, such as *RHO1* itself, *RHO2, MSS4, MLP1*, a truncated form of *CDC24*, and *RGD2*. By analysis of nine RhoGAPs, we further found that either deletion of a Rho1p GAP (*SAC7* or *LRG1*) or overexpression of a Cdc42p GAP (*RGA1, BEM3*, or *RGD2*) suppressed the *cyk3Δ hof1Δ* growth defect. Consistent with this, depletion of the paxillin homolog Pxl1p, a protein that may function downstream of Cdc42p to negatively regulate Rho1p, also rescued this double mutant. Electron-microscopic analysis of some of these strains revealed that PS formation was not restored; instead, they had thick SS-like cell wall. Taken together, the evidence suggests that either activation of Rho1p or inactivation of Cdc42p leads to hyperdeposition of SS material, which increases the chance of successful (albeit not normal) cytokinesis. Interestingly, deletion of *SAC7* causes a synthetic growth defect in combination with *cyk3Δ*; because *cyk3Δ* itself causes slower PS formation accompanied by simultaneous formation of SS, Cyk3p may have a role in repressing SS formation until the completion of PS formation, perhaps through inactivation/inhibition of Rho1p.

1930/B393

Combining Chemical Genetics and RNAi to Identify Small Molecules That Target the Rho Pathway in Cytokinesis.

A. Castoreno[1,3], S. Krasteva[1,3], C. Valdez[1,3], M. Vokes[2], A. Carpenter[2], U. Eggert[1,3]; [1]Cancer Biology, Dana Farber Cancer Institute, Boston, MA, [2]Imaging Platform, Broad Institute, Boston, MA, [3]Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA

Rho GTPases are key regulators of cytokinesis, the last step in cell division where daughter cells physically separate. The underlying mechanisms by which Rho coordinates cleavage furrow formation, ingression and cytokinesis completion remain poorly characterized. To address this question, we have developed a high-throughput phenotypic screen to discover Rho pathway-specific small molecules using a combination of chemical genetics and RNAi. Approximately 40,000 natural products and commercial compounds have been screened in Drosophila Kc167 cells and a number of promising candidates have been identified. Small molecules that target the Rho pathway will be important tools to dissect in more detail the role of Rho signaling during cytokinesis. In addition, altered Rho signaling has been linked to tumor development and progression in several human cancers, including breast, pancreas, and liver cancers. Therefore, Rho pathway-specific compounds from our screen may also catalyze therapeutic studies.

1931/B394

Differential Recruitment of Cytokinetic Factors in Cells Containing Monopolar Spindles.

S. Shrestha, J. Eyer, L. Wilmeth, C. Shuster; Biology MSC3AF, New Mexico State University, Las Cruces, NM

Initiation of cytokinesis, the terminal event of cell division, is marked by the equatorial assembly of an actomyosin contractile ring that constricts to produce two daughter cells. Central to this process is the equatorial activation of the small GTPase, RhoA, which is accomplished through the actions of the centralspindlin complex and the RhoGEF, Ect2. Multiple signaling molecules have been implicated in this process, including Aurora B and Plk1. Both kinases are recruited to the cell equator during anaphase, and in order to investigate the respective roles of these molecules at the cell equator, we studied their mobilization in cells containing monopolar spindles that are forced

into mitotic exit. Examination of CPC- and centralspindlin components revealed that upon the induction of mitotic exit, MKLP-1, Aurora B and the CPC were rapidly recruited to the site of ectopic furrowing in a microtubule-dependent manner. Similarly, Ect2 and RhoA were recruited to the polarized site of ectopic furrowing. In contrast, Plk1 was retained on kinetochores and centosomes, and failed to mobilize to the site of furrowing in monopolar cells forced into mitotic exit. Plk1 depleted cells arrest in mitosis with monopolar spindles, and when forced into mitotic exit, efficiently recruited Aurora B, MKLP-1, Prc1 (the docking molecule for Plk1 at the spindle midzone) and RhoA to the site of ectopic cleavage. Together, these results suggest that Aurora B and Plk1 have differential requirements for localization to the cleavage plane, and may play partially redundant roles in the activation of RhoA during cytokinesis.

1932/B395

Bub2 Regulation of Cytokinesis and Septation in Budding Yeast.

S. Park, A. Cable, K. Stockstill, K. Shannon; Biological Sciences, Missouri University of Science and Technology, Rolla, MO

The mitotic exit network (MEN) is required for events at the end of mitosis such as degradation of mitotic cyclins and cytokinesis. Bub2 negatively regulates the MEN GTPase Tem1, and Bub2 is required to delay the cell cycle in response to mispositioned spindles. It has been proposed that Bub2 is also required to prevent premature actin ring formation. We tested this hypothesis by comparing the timing of actin ring formation in wild type, bub2Δ, mad2Δ, and bub2Δ mad2Δ cells both with and without nocodazole. Cells were synchronized using α factor, released into media with or without nocodazole, fixed at numerous time points, and stained for actin to determine the timing of actin ring formation. There was no difference in the timing of actin ring formation between wild type and mutant cells without nocodazole. In the presence of nocodazole, both bub2Δ and mad2Δ cells formed rings after a delay of the same duration. bub2Δ mad2Δ cells formed rings in nocodazole at the same time as they did without nocodazole. Together, this data indicates that Bub2 does not have a role in delaying actin ring formation. Previous data suggested that Tem1 is required for actomyosin ring contraction. To determine if Bub2 has an effect on contraction through its regulation of Tem1, we used live cell imaging of Myo1-GFP in a bub2Δ strain. We found a significant decrease in the total time of contraction and increase in rate of contraction compared to wild type cells. We also examined myosin contraction using Myo1-GFP in cells overexpressing Bub2. Surprisingly, this also led to a significant increase in the rate of contraction, as well as a septation defect. Our data suggests that the overexpression of this Bub2 fusion protein acts as a dominant negative.

1933/B396

Phosphorylation of MyoGEF on Thr-574 by Plk1 Promotes Myogef Localization to the Central Spindle.

M. Asiedu[1], D. Wu[1], F. Matsumura[2], Q. Wei[1]; [1]Department of Biochemistry, Kansas State University, Manhattan, KS, [2]Dept of Molecular Biology & Biochemistry, Rutgers University, Piscataway, NJ

We reported previously that a guanine nucleotide exchange factor, MyoGEF, localizes to the central spindle, activates RhoA, and is required for cytokinesis. In this study, we have found that Plk1 (polo-like kinase 1) can phosphorylate MyoGEF, thereby recruiting MyoGEF to the central spindle as well as enhancing MyoGEF activity toward RhoA. The in vitro kinase assay shows that Plk1 can phosphorylate MyoGEF on threonine 574. Immunoprecipitation/immunoblot analysis demonstrates that mutation of threonine 574 to alanine dramatically decreases threonine phosphorylation of MyoGEF in transfected HeLa cells, suggesting that threonine 574 is phosphorylated in vivo. Consistent with these observations, immunofluorescence shows that Plk1 and MyoGEF colocalize at the spindle pole and central spindle during mitosis and cytokinesis. Importantly,RNAinterference-mediated depletion of Plk1 interferes with the localization of MyoGEF at the spindle pole and central spindle. Moreover, mutation of threonine 574 to alanine in MyoGEF or depletion of Plk1 by RNA interference leads to a decrease in MyoGEF activity toward RhoA in HeLa cells. Therefore, our results suggest that Plk1 can regulate MyoGEF activity and localization, contributing to the regulation of cytokinesis.

1934/B397

Two Phosphopeptide Binding Modules Act Sequentially to Activate RhoA during Cytokinesis.

B. A. Wolfe[1], T. Takaki[2], M. Petronczki[2], M. Glotzer[1]; [1]Molecular Genetics and Cell Biology, The University of Chicago, Chicago, IL, [2]Cell Division and Aneuploidy Laboratory, Cancer Research UK London Research Institute, Hertfordshire, United Kingdom

To complete cell division with high fidelity, chromosome segregation and cytokinesis must be strictly coordinated. In animal cells, each of these processes is specified by microtubule-based structures: microtubules of the mitotic spindle facilitate chromosome movement to the cell poles while those of the central spindle help to position the cleavage furrow. By capitalizing on its localization to these structures, the mammalian Polo-like kinase Plk1 functions as a critical intermediary, performing essential functions in chromosome segregation and in specification of the cleavage plane. Inhibition of Plk1 kinase activity during anaphase blocks the localized activation of the small GTPase RhoA at the equatorial cortex and prevents cleavage furrow formation. While Plk1 recruitment to the central spindle requires phospho-dependent association of its C-terminal Polo Box Domain (PBD) with the microtubule-associated protein (MAP) Prc1, a critical target(s) of its kinase activity necessary for localized RhoA activation has not been identified. Using a combination of biochemical and cell biological assays, we describe the molecular mechanism by which Plk1 directs RhoA activation at the equatorial cortex. At the central spindle, Plk1 phosphorylates the non-catalytic N-terminus of the RhoGAP HsCyk-4, creating a phospho-epitope recognized by the BRCA1 C-terminal (BRCT) repeats of the RhoGEF Ect2. Preventing HsCyk-4 phosphorylation abrogates association with Ect2 in vitro and in vivo and disrupts cleavage furrow formation. Plk1 phosphorylation of HsCyk-4 does not depend upon PBD association with HsCyk-4 or Ect2. Instead, PBD tethering to Prc1 places the kinase in close proximity to permit robust phosphorylation of HsCyk-4. Together, our data point to a regulatory circuit in which RhoA is activated at the equatorial cortex through the sequential action of two phosphobinding modules.

1935/B398

How Rho1 Is Concentrated at the Division Site to Enable Cytokinesis.

S. Yoshida, S. Bartolini, D. Pellman; Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA

The small GTP binding protein Rho1 (RhoA) is essential for cytokinesis from yeasts to human. A molecular understanding of how cell cycle signals activate Rho1 at the division site is beginning to emerge. Polo kinase binds and phosphorylates Rho GEFs (Guanine nucleotide

exchange factors) and targets GEFs to the division site, enabling Rho recruitment, activation and contractile ring assembly (CAR, Yoshida et al, Science 2006). However, the mechanisms responsible for Rho enrichment at the division site remain a fundamental open question. Here, we demonstrate that budding yeast sequentially utilizes two mechanisms to concentrate Rho1 at the mother-bud neck. The first mechanism involves GEF binding to Rho1 in its GDP or unliganded state. This mechanism is essential for CAR assembly. We were able to test this hypothesis by generating viable yeast lacking all Rho1 GEFs. Thus, Rho function in cytokinesis requires Rho1 to cycle between GDP and GTP-bound states; GTP-fixed mutant Rho1 is a loss of function with respect to CAR assembly. A second mechanism, likely involving phospholipids binding to the Rho1 polybasic sequence, is utilized late, around the time of abscission. These findings comprehensively define the targeting mechanisms Rho1 essential for cytokinesis and novel activation mechanisms.

1936/B399

A Possible Mechanism for Midzone Alignment in Cytokinesis.

C. Hu, C. Field, T. Mitchison; Systems Biology, Harvard Medical School, Boston, MA

During cytokinesis, correctly positioning the contractile ring and cleavage furrow on the cortex mostly depends on the stable signals from microtubules to the cortex. Different to mitosis, microtubules in cytokinesis are relatively less dynamic, and organize into the midzone in an antiparallel manner. Proteins required for the signaling from microtubules to the cortex, such as PRC1, Centralspindlin, and Rho-GEF Ect2, all localize on the central region of midzone where microtubule are interdigitating overlapped. In order to precisely position a narrow furrow region on the broad equatorial cortex, localizations of these proteins should be tightly regulated, thus to provide a stable and robust signal. After the onset of cytokinesis, these furrow-required cytokinetic proteins are initially diffused along midzones, but gradually concentrated to the center with time. Despite they associate with separated microtubule bundles, eventually these proteins would be positioned on the same plane in the Z-axis, signaling the cortex to build up a furrow on the same plane. We are interested in how these proteins on different midzone bundles communicate to one another, coherently localizing on the same plane without direct interaction. Here we address this question by disrupting different proteins in cytokinesis. Our preliminary data suggest this coherent positioning mechanism might involve a local gradient of molecules, and should be independent to actin and myosin systems.

1937/B400

Inhibition of Cortical Myosin Recruitment by Astral Microtubules.

Y. Tse, M. Glotzer; Department of Molecular Genetics and Cell Biology, University of Chicago, Chicago, IL

Cytokinesis, the final step of cell division in which the two daughter cells are physically separated, is driven by constriction of the contractile ring consisting of actin and non-muscle myosin. The position of the contractile ring is determined by the spindle that leads to the local activation of RhoA which, in turn, promotes actin polymerization and myosin activation. Two mechanistically distinct pathways redundantly regulate cleavage furrow formation. In the first pathway, central spindle microtubule bundles positively promote local recruitment of myosin; whereas in the second pathway, astral microtubules locally inhibit the accumulation of cortical myosin. In embryos furrowing solely via the astral pathway, cortical myosin foci are recruited upon anaphase onset, and coalesce to form large myosin foci concentrated in the division plane. These large foci do not accumulate in near regions of high microtubule density. The molecular mechanism of this local inhibition of cortical myosin is incompletely understood. Here, we have used high-resolution imaging and 3D reconstruction to examine the relationship between cortical myosin and microtubules. We find that most of the large myosin foci do not appear to interact with astral microtubules — they rather accumulate at a distance from them. We do, however, detect a subpopulation of myosin foci that do associate with microtubules, suggesting that microtubules could directly influence cortical myosin behavior. Using photobleaching techniques, we cannot detect a major difference in the turnover of myosin foci in regions with vastly different microtubule densities. These data indicate that astral inhibition regulates cortical myosin recruitment, but not its stability. We will present a progress report on the identification of the molecular basis of local regulation of cortical myosin.

1938/B401

Lats2 Is Essential for the Final Stage of Cytokinesis in Mammalian Cells.

P. Murugan, D. McCollum; Molecular Genetics and Microbiology, Umass Medical School, Worcester, MA

Mammalian Lats2 kinase is a homolog of the Drosophila tumor suppressor protein Lats/Warts. In Drosophila, Lats coordinates cell proliferation and cell death as a component of the hippo signaling pathway. Several known molecules of the Lats pathway appear to be evolutionary conserved in mammalian cells, and dysregulation of many of these proteins has been implicated in genesis of human solid tumors. Lats protein is a homolog of Dbf2 of S. cerevisiae and Sid2 of S. pombe, which localize to spindle pole bodies (SPB) and regulate mitotic exit and cytokinesis. Lats2 in mammalian cells localizes to centrosome through out the cell cycle and to the midbody ring during cytokinesis. Mutant cells derived from Lats2 knock out mouse embryos shows mitotic defects, including centrosome fragmentation and cytokinesis failure. However the exact role of Lats2 in mitotic exit and cytokinesis remains unknown. Here, we show that Lats2 is essential for normal completion of cytokinesis. Systematic deletion analysis reveals that the non-kinase domain of Lats2 is essential for its localization to the centrosome and midbody. RNAi mediated knock down of Lats2 leads to failure of abscission, the final stage of cytokinesis, and increases the percentage of cells that are connected by a thin cytoplasmic bridge. However it does not affect the localization of the chromosomal passenger complex to the midbody. The observed phenotype is similar to the knockdown of Centriolin, whose homolog in S.pombe called cdc11, binds directly to the Lats2 homolog Sid2. Together these results raise the possibility that these proteins may function as part of an evolutionarily conserved signaling network regulating cytokinesis.

1939/B402

Characterization of NDR Kinases in Dictyostelium Discoideum.

P. Kastner, M. Schleicher, A. Mueller-Taubenberger; Institute for Cell Biology, Ludwig Maximilians University, Munich, Germany

NDR (nuclear Dbf2-related) kinases are a subgroup of the AGC (protein kinase A/G/C-like) class of protein kinases. The genome of *Dictyostelium discoideum* encodes four NDR group kinases, NdrA, B, C and D. Sequence comparisons indicate that NdrA and B correspond

to human kinases NDR1 and 2 that were shown to play a role in centrosome duplication. NdrC and D are related to the large tumor suppressors LATS1 and 2. In addition to the different NDR kinases, in *D. discoideum* three potential NDR activators of the Mob1 group, MobA, B and C are expressed. Our current working hypothesis is that these different Mob1 proteins specifically activate distinct NDR kinases. In *Drosophila melanogaster*, homologs of NDR kinases play a role in development. In *Schizosaccharomyces pombe* a NDR kinase (Sid2p) is part of the septation initiation network (SIN), which is important for the final stages of cytokinesis. We recently provided evidence for a SIN-homologous pathway involved in the regulation of cytokinesis in *D. discoideum*. Upstream components of this pathway include a polo-like kinase (PLK), a GAP (Bub2), a GTPase (Spg1), and a central Ser/Thr-kinase (SepA, a homolog of the SIN-kinase Cdc7p). The effectors downstream of SepA are not specified yet, but may include members of the Ste20-like and NDR group of kinases. Therefore, we started to explore the *D. discoideum* NDR kinases and test their potential involvement in cytokinesis. NdrA was shown to localize to the centrosome. Deletion of the *ndrA* gene results in reduced growth. NdrA mutant cells have defects in phagocytosis, but cytokinesis is unaffected. NdrB kinase also localizes to centrosomes. By immunoprecipitation studies and tandem affinity purification (TAP), MobB that localizes to the cytoplasm and the nucleus, was found to interact with NdrB. Our data suggest that NdrB together with MobB may be components downstream of SepA in the SIN-related pathway of *D. discoideum*.

1940/B403

Timely Septation Requires SNAD-Dependent Spindle Pole Body Localization of the Septation Initiation Network Components in the Filamentous Fungus Aspergillus Nidulans.

C. Zeng, J. Kim, R. Shao, B. Liu; Department of Plant Biology, University of California, Davis, CA

In the filamentous fungus *Aspergillus nidulans*, cytokinesis/septation is triggered by the kinase cascade of the septation initiation network (SIN) which first appears at the spindle pole body (SPB) during mitosis. The novel coiled-coil protein SNAD is associated with SPB, and is required for timely septation, and conidiation. We have determined that SNAD acted as a scaffolding factor that was required for the localization of SIN proteins of SIDB and MOBA to SPB. Another scaffolding factor SEPK, whose localization at SPB was dependent on SNAD, was also required for SIDB and MOBA localization to SPB. In the absence of either SEPK or SNAD, SIDB/MOBA successfully localized to the septation site, indicating that their prior localization at SPB was not essential for their later appearance at the division site. Our results suggested that through SEPK, SNAD mediated the interaction between SIN components and cell cycle regulators at SPB. Unlike their functional counterparts in fission yeast, SEPK and SNAD were not required for vegetative growth except for timely septation. Furthermore, hyperactivation of the SIN pathway by downregulation of the GAP components suppressed the phenotype of poor conidiation due to the loss of SNAD. Therefore, we concluded that SPB localization of SIN components was not essential for septation per se, but critical for septation to take place in a timely fashion. In addition, timely execution of septation was a prerequisite of conidiation. This work was supported by the National Science Foundation.

1941/B404

The Sin Kinase Sid2 Regulates Cytoplasmic Retention of the Cdc14-Like Phosphatase Clp1 in S. Pombe.

C. Chen[1], A. Feoktistova[3], J. Chen[3], Y. Shim[2], D. M. Clifford[3], K. Gould[3], D. McCollum[1,2]; [1]Interdisciplinary Graduate Program, UMass Medical School, Worcester, MA, [2]Molecular Genetics and Microbiology, UMass Medical School, Worcester, MA, [3]Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, TN

The septation initiation network (SIN) in the fission yeast Schizosccharomyces pombe plays an essential role in cytokinesis. The SIN is required in late mitosis to maintain assembly of the actomyosin ring and promote septum formation. The Cdc14-like phosphatase Clp1 functions together with the SIN to coordinate cell and nuclear division cycles. In interphase, Clp1 localizes to the nucleolus where it is thought to be sequestered. At the onset of mitosis, Clp1 is released from the nucleolus. The SIN becomes activated at the end of anaphase and functions together with Clp1 to promote cytokinesis. Clp1 promotes SIN signaling by antagonizing CDK activity, and the SIN acts to keep Clp1 in the cytoplasm. It has been shown that the cytoplasmic retention of Clp1 requires both SIN signaling and the 14-3-3 protein Rad24. In vivo, the most downstream SIN component, the NDR-family kinase Sid2, is required for direct binding between Clp1 and Rad24. Because Rad24 is restricted to the cytoplasm, its binding to Clp1 may retain the Clp1-Rad24 complex in the cytoplasm. We show using recombinant proteins in vitro that Clp1 will only bind Rad24 when it has been pre-phosphorylated by Sid2. We identified six sites of Sid2 phosphorylation on Clp1 using mass spectrometry. Mutation of these six phosphorylation sites disrupts Clp1 localization at the end of mitosis (telophase). While wild-type Clp1 remains in the cytoplasm until cytokinesis is completed, the mutated Clp1 (Clp1-6A) returns to the nucleolus before completion of cytokinesis. Loss of Clp1 from the cytoplasm in telophase renders cells sensitive to perturbation of the actomyosin ring, but does not affect other functions of Clp1. Together these results describe a molecular mechanism by which the SIN causes retention of Clp1 in the cytoplasm in late mitosis. Because all of the components of this pathway are present in mammalian cells, we propose that this might be a broadly conserved mechanism for cell cycle regulation.

1942/B405

From Midbody Protein-Protein Interaction Network Construction to Novel Regulators in Cytokinesis.

T. Chen[1], Y. Liu[1], S. Lee[2], J. Lai[3], C. Kao[1], C. Huang[1,2]; [1]Institute of Clinical Medicine, National Yang-Ming University, Taipei, Taiwan, [2]Department of Computer Science and Information Engineering, National Taiwan University, Taipei, Taiwan, [3]Department of Life Science, Fu-Jen Catholic University, Taipei, Taiwan

Midbody, a transient organelle-like structure, is known as central for abscission and is indispensable for termination of cytokinesis. Inappropriate formation of midbody significantly affects cytokinesis progression and causes multinuclear cells, the characteristic of many cancers. Here, we used the midbody proteome inventories to construct the potential midbody protein-protein interaction (PPI) network. To delineate novel regulators participate in cytokinesis, the z-score, a standard statistic score, rather than hub degree was implemented to prioritize the novel hubs. Of these hubs, KIAA0133, SEPT1, KIAA1377, and CRMP-1, were localized to midbody, whereas HTR3A and ICAM2 were associated with cleavage furrow as examined by mmunofluorescence. Knockdown of SEPT1, KIAA1377, or CRMP-1 resulted in increasing the numbers of cytokinesis defect cells, suggesting these newly identified hubs play critical roles in cytokinesis progression.

Moreover, ectopic expression of CRMP-1 mutant in which Aurora-A phosphorylation sites have been replaced with Ala results in a cytokinesis defect, similar to the phenotype of CRMP-1 siRNA, consisting with the well-known progression of mitotic events relying on protein phosphorylation. This sub-proteome network construction not only sheds light on the intimate interactions of the midbody proteomes, but also prioritizes novel hubs or protein complexes that may govern the process of cytokinesis. Together, these integrated bioinformatics and empirical approaches may provide a foundation to study PPI network of a given organelle or disease state.

1943/B406

Translational Regulation of Cell Wall Biosynthesis Genes in a Myo1δ Mutant Strain of the Budding Yeast Saccharomyces Cerevisiae.

M. E. Rivera-Ruiz, J. Rodriguez-Medina; Biochemistry, School of Medicine Medical Sciences Campus , University of Puerto Rico, San Juan, PR

Post-transcriptional control is critical in regulating a variety of physiological processes including development, cell differentiation, protection of cells from external damage and regulation of metabolic pathways. Cells with myosin II deficiency undergo a myosin II independent cytokinesis mechanism that involves increased chitin deposition at the cell wall by chitin synthase III (CSIII). To determine if these phenotypes were related to changes in gene expression, we have previously determined the global transcriptional profile of *myo1Δ* strains. These studies corroborated an increase in transcription of Slt2p(Mpk1p)mRNA a late effector in the *PKC1* cell integrity pathway. Many cell wall protein mRNAs are regulated transcriptionally by the activation of this pathway. However, the response of eukaryotic cells to stress also occurs at posttranscriptional levels. The objective of this study is to assess the range of mRNAs related to cell wall integrity regulated at the translational level in *myo1Δ* strains. To better understand the range of mRNAs affected by translational regulation, total RNA from whole ribosomal pellets was extracted and cRNA hybridized to yeast oligonucleotide microarrays. Transcriptionally regulated mRNAs were excluded from this analysis. A total of 1,271 differentially regulated genes ($p \leq 0.018$) were identified in the *myo1Δ* strains of which 43 were cell wall biosynthesis genes. A polyribosome profile analysis was performed on mRNAs of the CSIII expression pathway. Our preliminary results showed that *CHS3* and *CHS4* mRNAs were reduced in the heavier polyribosomes and increased in the 40S and 80S fractions in *myo1Δ* strains, suggesting that these mRNAs are less efficiently translated in this mutant. Supported by MBRS-SCORE (1SC1AI081659-01), MBRS-RISE (R-25GM61838) with partial support from NCRR-RCM1 (G12RR-03051).

1944/B407

Role of Cyk3p-Hof1p Interaction in Actomyosin-Ring-Independent Cytokinesis in Saccharomyces Cerevisiae.

M. Wang, R. Nishihama, M. Onishi, J. Pringle; Department of Genetics, Stanford University School of Medicine, Stanford, CA

In the budding yeast *Saccharomyces cerevisiae*, cytokinesis is achieved by the coordinated actions of the actomyosin contractile ring (AMR), re-organization of the plasma membrane, and formation of the septal cell wall. Formation of the AMR depends on Myo1p, the sole type II myosin, but *myo1Δ* cells lacking the AMR are viable in most strain backgrounds, indicating that the AMR is not essential for cytokinesis. Indeed, some *myo1Δ* cells form nearly normal cleavage furrows and primary septa (PS). To investigate the mechanisms of AMR-independent cytokinesis, genetic screens were performed using *myo1Δ* mutants; they identified *IQG1* (encoding the only *S. cerevisiae* IQGAP), *CYK3* (encoding a protein that contains an SH3-domain and a possible transglutaminase domain), and *HOF1* [encoding a member of the PCH (Pombe Cdc15 homology) protein family, members of which have been suggested to function in promoting membrane deformation by means of their "F-BAR" domains]. These three proteins all localize to the division site during cytokinesis. Iqg1p is required for formation of both the AMR and the PS. Overexpression of *CYK3* can suppress the *iqg1Δ* growth defect without restoring the AMR, suggesting that Cyk3p acts downstream of Iqg1p to promote AMR-independent cytokinesis. By two-hybrid analysis using Cyk3p as bait, we have identified a physical interaction with Hof1p, which was unexpected because previous genetic evidence (synthetic lethality of the *cyk3Δ hof1Δ* double mutant) had suggested that the two proteins functioned in parallel pathways. Co-immunoprecipitation assays have confirmed the Cyk3p-Hof1p interaction and suggested that this interaction occurs specifically around the time of cytokinesis. Further two-hybrid analyses using fragments of Cyk3p or Hof1p have indicated that the interacting domains are a proline-rich motif in Cyk3p and the SH3-domain in Hof1p. Subsequent mutational analyses have shown that the interacting domains in both proteins are not required for their bud-neck localization but are essential for their function in AMR-independent processes of cytokinesis.

Signal Transduction II (1945 – 1971)

1945/B408

Elucidating the Mechanism of Colony Formation in Pseudomonas Aeruginosa PA.

C. Okgebe[1], L. Dietrich[2,3], D. K. Newman[2,3]; [1]Biology, Howard University, Washington, DC, [2]Earth and Planetary Sciences, MIT, Cambridge, MA, [3]HHMI, MIT, Cambridge, MA

Historically, multicellularity has been discussed predominantly in the context of eukaryotic organisms; the fact that communities of bacteria also exhibit this property is often underappreciated. Individual bacteria within a community excrete small molecules that are taken up by other bacteria and transduce information. Biofilm-forming bacteria such as Pseudomonas aeruginosa produce small molecules called phenazines. Phenazine derivatives are heterocyclic pigments that regulate redox homeostasis in the biofilms by shuttling electrons between the organism and the environment. P. aeruginosa forms biofilms in response to environmental cues such as nutrient paucity and oxygen deficiency. However, the mechanisms by which P. aeruginosa senses environmental and endogenous signals, how these signals are mediated via signal transduction cascades, and the mechanisms for cellular response are unknown. P. aeruginosa colonies, grown on agar amended with Congo red and Coomassie blue, exhibit dramatic phenotypic changes when phenazine production is repressed or over-expressed. We performed a colony morphology assay on a library of approximately 6000 transposon insertion mutants to identify genes involved in signaling pathways that modulate colony structure. Different phenotypes were characterized and the genes responsible were annotated. Using a recombinase system, double mutants were made with mutations occurring at the phenazine gene site and other gene sites that showed

desirable phenotypes simultaneously. Our results suggest that PA1106, a putative sensor, is involved in the organization of these multicellular communities.

1946/B409

Bacteriophage-Expressed Soluble Substrates for Protein Kinases.

M. Arcand, A. Marcil, S. Dahan, F. Lipari, P. Roby, M. Bielefeld-Sévigny; Bio-discovery, PerkinElmer LAS, Montréal, QC, Canada

The human kinome comprises nearly 520 protein kinases that serve as key mediators of processes such as growth, metabolism, inflammation, cell division and apoptosis. Because kinases play crucial regulatory roles in so many processes, they are often linked to disease and represent attractive pharmacological targets. Despite extensive efforts, there remain numerous "orphan" kinases for which no substrate has yet been identified. Our goal is to develop a screening assay for the identification of new substrates for protein kinases. We turned towards available M13 and T7 phage display systems since these bacteriophages exhibit fast and simple replication, and can be used as expression systems that physically link a protein target with its DNA coding sequence. We therefore constructed various M13 and T7 phages harbouring consensus phosphorylation sequences for the tyrosine kinase c-Src and the serine/threonine kinase Akt1. Normalized phage populations were used as substrates in kinase assays performed in well plates. The normalization of phage numbers was carried out using AlphaLISA® beads coupled to antibodies directed against bacteriophage structural proteins. This method is an effective substitute for the tedious, yet conventional phage titration. In a similar bead assay setting, anti-phosphorylated consensus antibodies were used to determine the phosphorylation of phage-expressed substrates following kinase assays. We found the substrate constructs expressed at the surface of M13 and T7 bacteriophages to be phosphorylated by the kinases, whereas non-phosphorylatable mutants were not. These data suggest that phosphorylation occurs specifically on the expressed constructs and not on bacteriophage structural proteins. Such tailored phages represent a simple, fast, self-replicative and inexpensive way of determining the kinase activity. Ultimately, this assay platform should enable us, not only to measure phosphotransferase activity, but also to identify new targets for virtually any protein kinase.

1947/B410

Towards an SH2 Interactome: Using a pY-Specific Y2H System to Identify and Analyse Novel Lyn Kinase Signalling Pathways Involved in Erythropoiesis and Cancer.

E. Ingley[1,2], L. S. Tie[1,2], S. Murphy[1,2], D. J. McCarthy[1,2], C. J. Payne[1,2], S. P. Klinken[2]; [1]Cell Signalling Group, Western Australian Institute for Medical Research, Perth, WA, Australia, [2]Laboratory for Cancer Medicine, Western Australian Institute for Medical Research, Perth, WA, Australia

We have developed a phosphotyrosine-specific yeast two-hybrid system and used it to identify molecules that interact with phosphorylated substrates of the Src family kinase (SFK) Lyn. Using this method has resulted in the identification of new Lyn and other SFK regulatory mechanisms and novel pathways that emanate from SFKs. We show that the Lyn substrate Csk binding protein (Cbp) binds Lyn via a high affinity pY-SH2 interaction. Lyn phosphorylates Cbp after activation on multiple sites, including a site that recruit Lyn's negative site Y508 phosphorylating kinase, Csk/Ctk, allowing down regulation of Lyn's kinase activity. We also identified the SH2 domain of suppressor of cytokine signaling-1 (SOCS1) as binding specifically to phosphorylated Y314 of Cbp. We show SOCS1, which is transcriptionally induced by Lyn kinase activity, associates with Cbp to allow the ubiquitination and subsequent proteasomal degradation of Lyn. Further, other pY-motifs of Cbp interact with SH2 containing mediators of the Akt/PKB pathway. Our system has also identified novel oncogenic substrates of Lyn (Lyn associated cytoskeletal modulator, LACM) that link into the Vav and Nck pathways leading to major changes in cell shape by altering cytoskeletal dynamics. This new method is amenable to a high throughput screening strategy to identify pathways intersected by all the 120 SH2 domains present in the mammalian genome.

1948/B411

Bioinformatic Analysis of Arabidopsis Thaliana Chromosome I Kinome.

P. A. Karpov[2], A. Yemets[2], Y. B. Blume[1,2]; [1]Department of Genomics and Biotechnology, Institute of Food Biotechnology and Genomics, Kiev, Ukraine, [2]Deaprtment of Genomics and Biotechnology, Institute of Cell Biology and Genetic Engineering, Kiev, Ukraine

Protein kinases together with phosphatases participate in phosphorylation of numerous proteins in eukaryotic cells. Accordingly to rough prediction, in the Arabidopsis thaliana genome exist more then 1000 of protein kinase genes. We searched known A. thaliana protein kinases in the genomic and proteomic databases, such as Swiss-Prot/TrEMBL, Tair, GenBank, KEGG and GreenPhyl. Also we scanned the complete text of A. thaliana chromosomes with BLAST family programs using catalytic domains (TyrKc) of animal tyrosine kinases as search vectors. We performed revision the kinome for chromosome I, and identified 548 database records corresponding to protein kinases. After analysis of localization of sites of corresponding genes (Tair, NCBI MapViewer), and exclusion of duplicated and obsolete records, 280 protein kinases were designated to chromosome I. Applying SMART tool, we analyzed domain organization and domain composition of all marked proteins. Based on sequence homology, correspondence to Hidden Markov models (HMM) and to the patterns, we identified substrate specificity and margins of catalytic kinase domains. As far as protein kinases are heterogeneous in their domain organization and composition, further building of phylogenetic tree (Neighbors Joining, N-J) was carried out on the basis of results of multiplied alignment of their catalytic domains - the unique compulsory structure of all protein kinases. Visualization and analysis of phylogenetic tree was made with application of TreeView X and HyperTree software. During analysis and interpretation of results, we accounted information from databases and results of analysis of domain composition and function of individual domains. The information from Tair and GreenPhyl resources relating of classification of proteins was accounted also. As a result, we obtained annotated phylogenetic tree of the kinome for chromosome I. It was shown, that 212 protein kinases have dual substrate specificity (Tyr and Ser/Thr), and 67 are Ser/Thr protein kinases. Also, we identified that kinase At1g14000/F7A19.9 has catalytic domain which accordingly to HMM and sequence homology possesses by high level of similarity to classical animal tyrosine kinases.

1949/B412

Arabidopsis Phosphatome as a Target for Structural Bioinformatics Approaches.

A. Y. Nyporko[2,1], D. A. Samofalova[3,2], Y. B. Blume[1,2]; [1]Department of Genomics and Biotechnology, Institute of Food Biotechnology and Genomics, Kiev, Ukraine, [2]Department of Genomics and Biotechnology, Institute of Cell Biology and Genetic Engineering, Kiev, Ukraine, [3]Department of Cell Biology and Genetic Engineering, Kiev National University, Kiev, Ukraine

Protein phosphatases (PPs) being counterparts of protein kinases regulate a number of processes in eukaryotic cells through controlling of protein phosphorylation levels. Even a big progress in understanding the role of plant protein phosphorylation/dephosphorylation was achieved during last few years the structural features of plant PPs are still unexplored. In this study all PPs of Arabidopsis thaliana with completely deciphered amino acid sequences were estimated to carry out further structural structural three dimensional modeling of their spatial structure. Scanning of SwissProt databank has revealed 55 full-length sequences of PPs and phosphatase-like proteins from Arabidopsis which represent PP1, PP2A, PP2C, PPM (Ser/Thr phosphatases) and tyrosine phosphatases (PTP) families. Subsequent searching in ProteinDataBank for proteins found suitable matrixes for accurate spatial structure prediction of 48 analyzed PPs. Spatial structures of 2 phosphatases - kinase-associated PP (SwissProt access code P46014) and probable PTP At1g05000 (Q9ZVN4) - were resolved up to now with instrumental methods (NMR and X-ray crystallography, respectively, PDB access codes are 1MZK and 1XRI). 25 phosphatases have matrixes with amino acid sequence identity within a range from 50% to 82%. Maximal identity level between target and matrix amino acid sequences is proper for Ser/Thr-PPs PP2A-3 and PP2A-4 (SwissProt access codes P48578 and Q07100, best matrix is human PP2A core enzyme, PDB access code 2IE3). In addition, 21 revealed PPs have possible matrixes for 3D-structure prediction with sequence identity within a range from 30% to 48% taking into account that sequence lengths of these proteins (minimum 388 amino acid residues) is enough for correct prediction their 3D-structures. For 7 proteins, among them 6 are PP 2C poltergeist-like, development of correct spatial structure models is impossible yet. Therefore we can conclude Arabidopsis thaliana phosphatome in whole is very suitable for large-scale and accurate 3-D structural modeling despite some heterogeneity of amino acid sequences of individual PPs.

1950/B413

Down-Regulation of Protein Synthesis by Buckwheat Occurs by Inactivation of Eukaryotic Elongation Factor 2 and Potential Modulation of Phosphatase Activity.

D. M. Stringer[1], T. Marshall[1], C. G. Taylor[1], P. Zahradka[1,2]; [1]Human Nutritional Sciences, University of Manitoba, Winnipeg, MB, Canada, [2]Physiology, University of Manitoba, Winnipeg, MB, Canada

Eukaryotic elongation factor 2 (eEF2) regulates the elongation phase of protein translation by facilitating ribosomal movement along RNA. Activity of eEF2, represented by phosphorylation state, is determined by the balance between eEF2 kinase and protein phosphatase 2A. Insulin, through the action of mammalian target of rapamycin (mTOR) and p70S6k, decreases the activity of eEF2 kinase and thus activates eEF2. Buckwheat (BW) is a natural plant product that contains many phytochemicals, including insulin-mimetic inositolphosphoglycans. We have previously demonstrated that both insulin and BW stimulate phosphorylation of mitogen-activated protein kinase p42/44 (MAPK); however, only insulin fully activates p70S6k. Thus, it was hypothesized that BW, despite the presence of insulin-mimetic compounds, does not stimulate protein synthesis. The objectives of the current study were to compare the effects of insulin and BW treatment on the phosphorylation of proteins involved in the eEF2 signaling pathway, as well as protein synthesis in H4IIE hepatoma cells. Cells were treated with insulin (250 nM) or a buckwheat concentrate (2.5% v/v) for 10 minutes, after which protein was isolated and phosphoproteins were measured using Western blotting. Both insulin and BW treatment resulted in mTOR phosphorylation; however, insulin lowered the amount of phospho-eEF2, while BW treatment resulted in higher levels of phosphorylated, inactive eEF2. The latter effect was ablated when cells were pre-treated with PD98059, a MAPK inhibitor, paradoxically suggesting that MAPK phosphorylation is required for the inactivation of eEF2 by BW. Furthermore, levels of phospho-eEF2 in BW-treated cells were 10-fold higher than in untreated cells, suggesting that BW also inhibits activity of eEF2 by phosphatase inhibition. Protein synthesis, measured as 3H-leucine incorporation, was elevated by treatment with insulin, but not with BW. Interestingly, co-treatment of cells with insulin and BW resulted in inhibition of insulin-stimulated protein synthesis, demonstrating that signalling pathways controlling protein synthesis elicited by BW are dominant over those stimulated by insulin.

1951/B414

Multi-Parametric Profiling of Reactive Oxygen and Nitrogen Species in Live Cells.

I. Lebedeva, H. Cox, M. Szczepanik, D. Shen, G. Schwartz, D. Gattica, W. F. Patton; R&D, Enzo Life Sciences, Farmingdale, NY

Free radicals and other reactive species play important roles in many human physiological and pathophysiological processes, including cell signaling, aging, cancer, atherosclerosis, various neurodegenerative diseases and diabetes. Recent emphasis on multi-color imaging in high-content screening has created renewed demand for easily measured, non-invasive, and non-disruptive cellular and molecular probes. To date, however, concerted efforts in developing such fluorescent probes for the profiling of reactive oxygen and nitrogen species (ROS/RNS), specifically tailored to working in concert with one another, has been limited in scope. No single fluorescent probe offers the necessarily rich analytical output required to comprehensively provide information on the generation of multiple ROS/RNS analytes generated within a cell. However, we demonstrate that through the combination of three judiciously chosen fluorescent probes, in combination with a battery of specific inhibitors and activators of ROS/RNS pathways, distinction among different reactive species is feasible in live cells. Simultaneous discrimination among superoxide, nitric oxide and peroxynitrite is demonstrated using a simple optical setup in wide-field fluorescence microscopy, to clearly distinguish among the three fluorescent reporter molecules. Fluorescence detection using a battery of fluorescent probes that respond with different selectivity profiles to various ROS/RNS offers an attractive approach for studying ROS and RNS in a noninvasive manner, with the ability to spatially and temporally resolve cellular signaling events.

1952/B415

Loss-Of-Function Mutations and Two-Furin Domain Derivatives Reveal Insights About R-Spondin2 Structure and Activity.

S. Li[1], T. Yen[2], Y. Endo[1], M. Klauzinska[3], B. Baljinnyam[1], B. Macher[2], R. Callahan[3], J. S. Rubin[1]; [1]Laboratory of Cellular & Molecular Biology, National Cancer Institute, Bethesda, MD, [2]Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, CA, [3]Mammary Biology and Tumorigenesis Laboratory, National Cancer Institute, Bethesda, MD

R-spondins (Rspos) potentiate Wnt/β-catenin signaling, an important pathway in embryonic development that is constitutively active in many cancers. However, the mechanism whereby Rspos enhance Wnt/β-catenin signaling is controversial. To address this and other questions concerning Rspo structure and function, we expressed full-length wild-type Rspo2 and derivatives corresponding to putative loss-of-function mutations in the *Rspo4* gene. Analysis of β-catenin protein and transcriptional activity in a SuperTopFlash reporter assay demonstrated that the Rspo2 mutants had markedly decreased potency relative to the wild-type protein. The diminished activity of Rspo2/C78Y and Rspo2/C113R was attributable to a defect in their secretion, while Rspo2/Q70R lacked intrinsic activity. Analysis of an Rspo2 derivative that contained only the two furin-like domains (Rspo2-2F) provided the first information about the disulfide-bonding pattern of this motif, which was characterized by multiple short loops and unpaired cysteine residues. Rspo2-2F synergized strongly with Wnt-3a in a β-catenin reporter assay, an effect that was correlated with an unusually sustained phosphorylation of the Wnt co-receptor, LDL receptor-related protein 6 (LRP6), as well as an increase in total and cell surface LRP6. Co-immunoprecipitation experiments involving full-length, wild-type Rspo2 and the Q70R point mutant indicated that they both associate with LRP6 and Kremens, suggesting that these and other interactions may be important for Rspo2 stimulation of β-catenin transcriptional activity.

1953/B416

Anti-Angiogenic Attributes of Paeonol Oxime in Human Endothelial Cells and HT 1080 Fibrosarcoma Cells and Associated Effects on PI3K/AKT, VEGF and MMP-2.

S. Lee, H. Lee, H. Lee, J. Lee, W. Koh, S. Kim; Oriental Medicine, Kyung Hee Univ, Seoul, South Korea

Paeonol oxime (PO) is a derivative of paeonol that was known to have anti-proliferative and anti-inflammatory activities. Since many anti-inflammatory agents emerged as potential therapeutics for angiogenesis related diseases, the present study investigated the anti-angiogenic attributes of PO in basic fibroblast growth factor (bFGF)-stimulated human umbilical vein endothelial cells (HUVECs) and highly angiogenic human HT1080 fibrosarcoma cells. PO inhibited the bFGF-induced proliferation (IC50 ~ 6 μg/ml) of HUVECs much better than paeonol (IC50 > 200 μg/ml). PO suppressed bFGF-stimulated migration and in vitro capillary differentiation (tube formation) of HUVECs in a concentration dependent manner. PO also disrupted the neovascularization in bFGF treated chick chorioallantoic membrane (CAM) in vivo. In the HT1080 cells, PO induced cytotoxicity with IC50 ~ 31 μg/ml, more effective than against non-proliferating HUVECs (IC50 ~ 65 μg/ml). PO decreased the expression of phosphatidylinositol-3-kinase (PI3K) and phosphorylation of its downstream target AKT without appreciable effect on extracellular signal regulated kinase (ERK). PO also attenuated vascular endothelial growth factor (VEGF) protein expression and secretion and downregulated matrix metalloproteinase (MMP)-2 at mRNA level in HT 1080 cells. Taken together, these findings suggest that paeonol oxime may be a potent angiogenesis inhibitor applicable to angiogenesis related diseases such as cancers via inhibition of PI3K/Akt signaling pathway and VEGF and MMP-2.

1954/B417

Shc Proteins Competitively Inhibit TGF-β -Induced Smad Signaling.

S. Smith, M. Lee; Center for Craniofacial Molecular Biology, University of Southern California, Los Angeles, CA

TGF-β signaling is canonically mediated by Smad proteins that are activated upon serine phosphorylation by TGF-β receptors. However, TGF-β receptors also possess a tyrosine kinase activity that enables them to directly bind and phosphorylate Shc adapter proteins, thereby initiating the well-characterized Shc signaling pathway leading to Erk MAP kinase activation. Since Shc and Smad proteins are both phosphorylated by the TGF-β receptor complex, these ligands may compete for the same kinases. We therefore hypothesized that Shc silencing would increase TGF-β induced Smad activation. Since all three Shc proteins (46^{Shc}, $p52^{Shc}$, and $p66^{Shc}$) are phosphorylated by TGF-β, we further hypothesized that suppressing individual Shc isoforms would also increase Smad activation. These postulates were tested by transfecting Mv1Lu mink lung epithelial cells with either $p66^{Shc}$ siRNA or a pan-Shc siRNA that silences all three Shc isoforms. Shc silencing was subsequently confirmed by Shc Western analysis. To assess Smad signaling, cells were co-transfected with the p3TP promoter-reporter construct and stimulated with 4 ng/ml TGF-β for 24 h. The cells were then lysed and the induced luciferase activity was quantitated by luminometry. To assess Smad2 phosphorylation, transfected cells were rested for 24 h, starved in serum-free medium overnight, and stimulated with 4 ng/ml TGF-β for up to 30 min. The cells were then lysed, resolved by SDS-PAGE, and subjected to phosphorylated Smad2 Western analysis. Pan-Shc silencing was associated with 50% increase in TGF-β stimulated p3TP activity while $p66^{Shc}$ silencing was associated with a 20% increase. Similarly, $p66^{Shc}$ and pan-Shc silencing both increased TGF-β-induced Smad2 phosphorylation. These results suggest that Shc proteins competitively inhibit Smad association with the TGF-β receptor complex, thereby regulating Smad signaling.

1955/B418

Lactoferrin Is a Potent Modulator of Chondrocyte Signal Transduction and Metabolism.

M. Huettinger[1], N. Brandl[1], S. Marlovits[2], P. Huettinger[3]; [1]Med.Chem, MUW/ZPPI, Vienna, Austria, [2]Department of Traumatology, General Hospital, Vienna, Austria, [3]Plastic Surgery, Landesklinikum St. Poelten, St. Poelten, Austria

Objective: Cytokines and blood borne mediators regulate chondrocyte metabolism which is crucial for cartilage maintainance. In OA and more so in RA, activation of granulocytes causes a considerable rise in the concentration of lactoferrin in synovial fluid. We here investigate whether consequences on signal transduction result and whether effects for the balance between catabolic and anabolic metabolism in chondrocytes result. Methods: Changes in signal transduction by lactoferrin were analysed in cultured chondrocytes by immunodetection of the phosphorylated forms of ERK1/2 and p38 MAP kinases and translocation os Smad. Expression levels alterations of MMP1, 2, 3, 13, aggrecan and of LRP were detected by semiquantitative RT-PCR. The proteolytic activity of metalloproteinases was ascertained by casein and gelatine zymography. The expression of the cell surface receptor for lactoferrin signalling, LRP1, in cartilage was assayed by immuno-histochemistry and by immunoblotting in chondrocyte cell fractions. Results: Lactoferrin stimulates proliferation of chondrocytes, comparable to TGF-beta, activated p38 30fold and ERK1/2 up to 5fold within minutes. Surprisingly, lactoferrin effectively induced Smad translocation to the cell nucleus, a signal pathway ascribed to TGF-beta receptor activation. Significant increases of mRNA levels of

catabolic indicators such as MMP1, MMP3 and MMP13 were observed in parallel. Aggrecan synthesis, suggestive for anabolism, was suppressed. LRP1, a signalling competent receptor for lactoferrin, was found in dedifferentiated chondrocytes which are positioned on the surface of articular cartilage. In deeper zone chondrocytes were negative for LRP1. Cultured cells in various differentiation states exhibited a like expression pattern. Conclusion: Lactoferrin acts mitogenic on chondrocytes and activates MMPs. It initiates SMAD translocation, thus affecting TGF signalling, in addition to MAP kinase activation. This implies a function as an early phase cytokine that enhances MMPs what leads to degradation of damaged tissue and prepares for reconstruction by stimulating proliferation of chondrocytes.

1956/B419

Taurine Chloramine Activates Nrf2 and Upregulates Heme Oxygenase-1 Expression in Macrophages.

J. Jang[1,2,3], S. Piao[1,3], M. Cho[1], Y. Wang[1,2], Y. Cha[2,3], C. Kim[1,2,3]; [1]Laboratory for Leukocyte Signaling Research, Inha University School of Medicine, Incheon 400-71, South Korea, [2]Department of Pharmacology and Toxicology, Inha University School of Medicine, Incheon 400-71, South Korea, [3]BK21 Program, Inha University School of Medicine, Incheon 400-71, South Korea

Taurine chloramine (TauCl) is a reaction product from taurine and HOCl produced by myeloperoxidase system, and is much less oxidizing or toxic than HOCl. TauCl has been shown to protect cells from inflammatory injury by inhibiting the overproduction of O2- and NO in the activated inflammatory cells. To determine the mechanism of cytoprotection provided by TauCl, we examined whether TauCl could enhance the expression of heme oxygenase-1 (HO-1). While taurine had no effect, TauCl upregulated the expression of HO-1 and increased HO activity in RAW 264.7 cells. TauCl by itself generated reactive oxygen species (ROS) and diminished total glutathione (GSH) level. TauCl increased the translocation of NF-E2-related factor 2 (Nrf2) into nucleus and enhanced the biding of Nrf2 to the antioxidant response element (ARE). This, in turn, was responsible for the upregulation of HO-1 expression. In summary, TauCl generates ROS, reduces cellular GSH level, and activates nuclear translocation of Nrf2 and its binding to ARE leading to increase the expression of HO-1. The increased HO activity resulting from upregulated HO-1 expression produces more CO and inhibits the heme dependent electron transfer in various enzymes such as NADPH oxidase and iNOS, and suppresses the production of O2- and NO, respectively. Collectively, TauCl plays an essential role in the adaptive cytoprotection of phagocytic cells by increasing HO-1 expression and HO activity, and thus by suppressing additional overproduction of NO and O2- occurring in the inflammatory lesions.

1957/B420

Angiotensin Ii Blockade Impair Advanced Glycation End Products-Induced Renal Tubular Hypertrophy Partly by Modulating Nitric Oxide Signaling.

J. Huang, Y. Huang; Biological Science and Technology, Chung Hwa University of Medical Technology, Tainan, Taiwan

The accumulation of advanced glycation end products (AGE) is a key mediator of renal tubular hypertrophy in diabetic nephropathy (DN). Angiotensin II (Ang II) and nitric oxide (NO) were involved in the progression of DN. In this study, we have examined the molecular mechanisms of Ang II blockade and NO responsible for inhibition of AGE-induced renal tubular hypertrophy. Cultured human renal proximal tubular cells, exposed to non-glycated BSA or AGE in the presence of angiotensin converting enzyme inhibitors (captopril and enalapril), AT1/AT2 receptor antagonists (losartan and PD123319), NO donor S-nitroso-N-acetylpenicillamine, or PKG activator 8-pCPT-cGMPs were assessed for Ang II and NO generation, activation of cGMP-dependent protein kinase (PKG) and mitogen-activated protein kinase (MAPK) signaling pathways, and cellular hypertrophy. AGE markedly enhanced Ang II generation and ACE expression (but not AT1 and AT2 receptor expression). ACEIs or AT1/AT2 receptor antagonists treatments significantly attenuated AGE-inhibited NO production, cGMP synthesis, and inducible nitric oxide synthase (iNOS)/PKG activation. The decrease in NO generation and iNOS activity may mediate by the receptor for AGE (RAGE) and the transforming growth factor-β1 (TGF-β1)-independent pathway. Moreover, AGE-induced the extracellular signal-regulated kinase (ERK)/c-Jun N-terminal kinase (JNK)/p38 MAPK activation were markedly blocked by ACEIs, Ang II receptor antagonists, NO donor, and PKG activator. The ability of Ang II blockade and the NO/PKG signaling to inhibit AGE-induced cellular hypertrophy was verified by the observation that captopril, enalapril, losartan, PD123319, SNAP, or 8-pCPT-cGMPs inhibited fibronectin, p21Waf1/Cip1, and RAGE expression. It is concluded that Ang II blockade significantly attenuated AGE/RAGE-enhanced activation of the ERK/JNK/p38 MAPK pathway and renal tubular hypertrophy partly through induction of the NO/PKG signaling.

1958/B421

Arachidonic Acid Independent of Eicosanoid Metabolites Stimulate Epithelial Cell Growth via NF-Kb and PI3-K/Akt Signaling Pathways.

L. D. Alexander; Natural Sciences, University of Michigan-Dearborn, Dearborn, MI

Arachidonic acid has been shown to be mitogenic for renal proximal tubular cells, but the mechanisms whereby it stimulated cell proliferation have remained largely unknown. The objective of these studies was to examine the effects of arachidonic acid on epithelial cell proliferation in rabbit renal proximal tubular cells. Our results demonstrate that treatment of cells with arachidonic acid caused rapid phosphorylation of AKT, and this effect was also abolished by inhibition of phosphatidylinositol 3-kinase (PI3-K). Moreover, selective inhibition of PI3-K and Akt blocked arachidonic acid- induced DNA synthesis. Arachidonic acid also stimulated the activity of NF-kappaB (NF-κB), and PDCT, an inhibitor of this transcription factor, completely blocked proximal tubular proliferation. Lastly, inhibition of arachidonic acid metabolic pathways had no effect on arachidonic acid-induced NF-κB activity, Akt phosphorylation or epithelial cell proliferation. These studies suggest that, in rabbit renal proximal tubular cells, the proliferative effects of arachidonic acid involve the PI3-kinase/Akt signaling pathways, and to some extent, the NF-κB pathway, and this effect is independent of eicosanoid biosynthesis.

1959/B422

Inhibition of NF-Kb by Caffeic Acid Decreases Cholangiocarcinoma Growth In Vitro and In Vivo.

G. Alpini[2], P. Onori[7], S. DeMorrow[3], E. Gaudio[4], R. Mancinelli[4,3], A. Franchitto[4], Y. Ueno[6], G. Carpino[10], J. Venter[3], J. Savage[3], D. Alvaro[5], M. Marzioni[8], G. Fava[8], S. Kopriva[9], H. Francis[1]; [1]Research and Medicine, Scott & White Hospital/TAMU COM, Temple, TX, [2]Research, Medicine and Systems Biology and Translational Medicine, Central Texas Veterans Health Care System, Scott & White and Texas A & M

Health Science Center College of Medicine, Temple, TX, [3]Medicine, Texas A&M HSC COM, Temple, TX, [4]Department of Human Anatomy, University of Rome "La Sapienza", Rome, Italy, [5]Gastroenterology, Polo Pontino, University of Rome "La Sapienza", Rome, Italy, [6]Division of Gastroenterology, Tohoku University Hospital, Aobaku, Sendai, Japan, [7]Department of Experimental Medicine, State University of l'Aquila, L'Aquila, Italy, [8]Gastroenterology, University Politecnico delle Marche, Ancona, Italy, [9]Research, Central Texas Veterans Health Care System, Temple, TX, [10]Dept Health Science, IUSM, University of Rome, Rome, Italy

Caffeic acid phenethyl ester (CAPE) inhibits the growth of various tumor cells. We have shown that CAPE inhibits the growth of the cholangiocarcinoma (CCH) cell line, Mz-ChA-1 (from gallbladder) by inhibition of NF-κB and cell death. The AIMS were to: (i) study the role of CAPE in growth regulation of other CCH cell lines; (ii) evaluate the effects of CAPE on two family members of NF-κB, NF-κB1 (p50) and RelA (p65); and (iii) evaluate the effect of CAPE *In Vivo* in nude mice. METHODS: Time/dose effect of CAPE (0-40 μM) on cell growth (by MTS assay, PCNA immunoblots and BrdU incorporation) was evaluated in numerous CCH cell lines (from intrahepatic bile ducts) and normal cholangiocytes for 48 hours. The effects of CAPE on NF-κB1 (p50) and RelA (p65) were studied in Mz-ChA-1 cells treated with CAPE (20 μM). *In Vivo* studies were performed in BALB/c nude (nu/nu) mice implanted subcutaneously with Mz-ChA-1 cells and subsequently treated with daily injections of vehicle (DMSO) or CAPE (10 mg/kg BW in DMSO) for 77 days. Subsequently, tumor latency, growth, apoptosis and expression of VEGF proteins and receptors were examined. The expression of NF-κB1 and RelA was evaluated in tumor lysates and sections. RESULTS: CAPE decreases the growth of all CCH cell lines, but has no effect on normal cells. PCNA and BrdU labeling confirmed CAPE-induced cell cycle decrease. By immunofluorescence and immunoblots, we found that CAPE-treatment inhibited the expression of NF-κB1 and RelA compared to basal. In *In Vivo* studies, tumor growth was decreased and latency was increased 2-fold in CAPE compared to vehicle-treated nude mice. In tumor samples, decreased cholangiocarcinoma growth by CAPE was coupled with increased apoptosis. No changes were seen in VEGF protein and receptor expression, whereas expression of NF-κB1 (p50) and RelA (p65) was decreased in tumor sections and lysates from CAPE-treated mice. Conclusion: CAPE decreases the growth of cholangiocarcinoma by inhibition of NF-κB, decreased cell cycle and apoptosis. These results indicate that CAPE could be an important therapeutic tool in the treatment of cholangiocarcinoma.

1960/B423

Modulation of Signal Specificity of the TGF-B Family Related to the Receptor Expression.

M. Funaba[1], M. Murakami[2], K. Ogawa[3]; [1]Laboratory of Nutritinal Science, Kyoto University Graduate School of Agriculture, Kyoto, Japan, [2]Laboratory of Molecular Biology, Azabu University School of Veterinary Medicine, Sagamihara, Japan, [3]Laboratory of Cellular Biochemistry, RIKEN, Wako, Japan

Members of the transforming growth factor-β (TGF-β) family signal via their specific type I receptor referred as ALK and type II receptor. The current model indicates that type I receptor serine/threonine kinase, which is activated by type II receptor, activates ligand-dependent receptor regulated (R)-Smad, i.e., activin/TGF-β pathway-specific R-Smad (AR-Smad: Smad2/3) or BMP pathway-specific R-Smad (BR-Smad: Smad1/5/8) through phosphorylation of C-terminal serines. Here we report phosphorylation and activation of both subclasses of R-Smad by a ligand stimulation, which relates to expression level of the receptors. Co-expression of constitutively active type I and type II receptors phosphorylated both subclasses of R-Smad in COS7 cells, irrespective of receptors in a ligand-independent manner. This was also verified by in vitro kinase assays. TGF-β1 and BMP-2 induced not only phosphorylation of the respective ligand-dependent R-Smad but also that of the different subclasses of R-Smad in untransfected B16 melanoma cells. In B16 cells, treatment with TGF-β1 increased expression of Id (inhibitor of differentiation), known genes regulated by BMP, whereas BMP-2 up-regulated PAI-1 (plasminogen activator inhibitor-1), an AR-Smad-regulated gene. Gene transcript levels of type I and type II receptors were generally higher in B16 cells than in HeLa and HepG2 cells in which treatment with TGF-β1 or BMP-2 induced phosphorylation of only expected R-Smad. These results indicate that the TGF-β family signals are modulated at the R-Smad phosphorylation level by expression level of the receptors, which partly explains a diverse effect of this ligand family.

1961/B424

The Type I TGF-β Receptor Is Covalently Modified and Functionally Regulated by Sumoylation.

J. Kang, E. Saunier, R. Akhurst, R. Derynck; UCSF, San Francisco, CA

Transforming growth factor (TGF)-β signaling plays important roles in cell growth, differentiation, apoptosis, development and tumorigenesis. Given the important roles in cell physiology, it is no doubt that TGF-β signaling could be regulated by other signaling pathways and post-translational modifications. For instance, the function of the Smad proteins, downstream mediators of TGF-β signaling, is controlled by phosphorylation, acetylation, ubiquitylation and sumoylation. Less, however, is known as to whether the TGF-β receptors themselves are regulated by post-translational modification, although they play key roles in transduction of TGF-β signal to the cytoplasm. We show here that type I TGF-β receptor, TβRI, is post-translationally modified by sumoylation in response to TGF-β and that its sumoylation requires the kinase activities of both TbβI and the type II TGF-β receptor, TβRII. We also demonstrate that the TβRI receptor is sumoylated on lysine 389 and that the TβRI sumoylation regulates the receptor function by facilitating the recruitment and phosphorylation of Smad3 protein and thereby modulates TGF-β-regulated transcription and cell growth inhibition. Furthermore, TβRI sumoylation enhances in vitro invasion and in vivo metastasis of Ras-transformed cell. Therefore, these results indicate that TβRI sumoylation plays an important role in modulating the function of the TGF-β receptors and helps define the cellular responses to TGF-β.

1962/B425

Effects of Protein Kinase C on TGFβ Dependent Signaling and Cell Migration.

A. Gunaratne, J. Di Guglielmo; University of Western Ontario, London, ON, Canada

Transforming growth factor beta (TGFβ) family members are involved in many aspects of cellular behavior and aberrant TGFβ signaling has been linked to various human cancers. The TGFβ pathway is largely regulated through endocytic processes, and TGFβ signal propagation is linked to receptor internalization and trafficking. The Protein Kinase C (PKC) family consists of several structurally related serine/threonine

kinases that have been implicated in modulating the endocytic processes of various receptors. Here we show that broad inhibition of PKC activity results in delayed TGFβ receptor degradation and alters TGFβ receptor trafficking in the early endosomal compartment. Inhibition of classical PKC isoforms results in delayed receptor degradation and trafficking but does not alter receptor compartmentalization in the early endosome. The inhibition of either broad or classical PKC activity extends Smad2 phosphorylation levels upon TGFβ stimulation. Furthermore, we have found that atypical PKC isoforms co-localize with the TGFβ receptors at the leading edge of migrating cells, and associate with the TGFβ receptors through the adaptor protein Par6. Pharmacological inhibition of this interaction with aurothiomalate reduces TGFβ dependant migration but does not affect TGFβ mediated epithelial to mesenchymal transition. Our results indicate that members of the PKC family may play different roles in regulating TGFβ receptor signaling and trafficking and potentially lead to the enhancement of TGFβ dependent migration of cancer cells.

1963/B426

Species-Dependent Ensembles of Conserved Conformational States Define the Hsp90 Chaperone Atpase Cycle.

D. R. Southworth, D. Agard; Biochemistry and Biophysics, UCSF, San Francisco, CA

The molecular chaperone Heat Shock Protein 90 (Hsp90) is the central player in a multicomponent complex that is required for the folding and activation of numerous proteins including nuclear receptors and cell-cycle kinases. By analogy to hsp60 and 70 class chaperones and GHKL superfamily members it is generally thought that Hsp90 transitions between two stable conformations in an ATP-dependent manner. Previous structural work from our lab suggested that for the E. coli Hsp90 ortholog, HtpG, the hydrolysis cycle is more complex involving at least three different conformational states. Using single particle negative-stain electron microscopy (EM), we have determined 3D reconstructions of HtpG:AMPPNP and yeast Hsc82:AMPPNP that agree with the yeast Hsp90:AMPPNP:p23 crystal structure, establishing that nucleotide binding alone triggers a well-conserved conformational change in which the amino-terminal domains are brought together. Also by 3D reconstruction we confirm the proposed model for a very compact HtpG:ADP state that is significantly different than the ATP state. Single particle matching methods reveal that both E. coli and yeast Hsp90 exists in a dynamic conformational equilibrium and that AMPPNP binding stabilizes but does not completely determine the closed state. Finally, crosslinking methods have allowed structural analysis of trapped, nucleotide-dependent conformational states of yeast Hsc82 and human Hsp90α and establish that all three states are universal. These data support a model where the chaperone cycle is conserved but the conformational equilibrium varies between species, implicating evolutionary tuning of the different states to meet the specific client protein and metabolic requirements of an organism.

1964/B427

Quantitative Implications of Scaffolding in Signal Propagation.

S. Chapman, A. Asthagiri; Chemical Engineering, California Institute of Technology, Pasadena, CA

Intracellular regulatory networks that control cell behaviors are remarkably intricate and involve numerous components. Amidst this apparent complexity is a key organizing feature: scaffolds. Protein scaffolds bind concomitantly to multiple components of a signaling pathway, thereby promoting signal transmission along a common physical “backbone”. To understand the quantitative effects of scaffolding, we engineered yeast cells with systematic variations in the expression level of an exemplar scaffold, Ste5. We show that signal throughput exhibits a biphasic dependence on scaffold concentration. Unexpectedly, wild-type Ste5 expression is sub-optimal and supports only half-maximal signal throughput, allowing regulatory flexibility for tuning signal flux by modulating scaffold concentration. We show that such modulations would not compromise signal quality as both background noise and signal fidelity are robust to 50-fold changes in scaffold amount. Thus, scaffolding may be a general mechanism for tuning signal throughput, without disrupting high-quality signal transmission.

1965/B428

Baicalein Suppresses Hypoxia Induced HIF1 Alpha Protein Accumulation and Activation through Inhibition of Reactive Oxygen Species and PI 3 Kinase Akt Pathway in BV2 Murine Microglial Cells.

K. Hwang, I. Kang; Kyung Hee University, Seoul, South Korea

Hypoxia induces an inflammatory activation of microglia during cerebral ischemia. The transcription factor of hypoxia inducible genes hypoxia inducible factor 1 (HIF1) is known to be involved in inflammation and immune response. Although baicalein (BE), a flavonoid, is shown to have anti inflammatory effects and attenuate ischemic injury, its action mechanism is not understood well. Thus, we examined effect of BE on hypoxia induced HIF1 activation and its signaling mechanism in BV2 microglial cells. BE inhibited hypoxia induced HIF1 alpha protein accumulation and HIF1 transcriptional activation. Consistently, BE suppressed hypoxia induced expression of hypoxia responsive genes, iNOS, COX2, and VEGF. We then showed that BE inhibited hypoxia induced phosphorylation of Akt but not that of ERK and p38. Moreover, BE inhibited hypoxia induced PI 3 kinase activation. Finally, we showed that BE inhibited hypoxia induced ROS generation, and an antioxidant N acetylcysteine reduced hypoxia induced HIF1 and iNOS protein expression and PI 3 kinase Akt activation in BV2 microglia. Taken together, these results suggest that BE suppresses hypoxia induced HIF1 protein and activation as well as expression of hypoxia responsive genes by inhibiting ROS and PI 3 kinase Akt pathway in BV2 microglia.

1966/B429

Signaling Pathways Induces by Oleic Acid to Proliferation in Breast Cancer Cells.

A. Soto-Guzman, L. A. Castro-Sanchez, N. Navarro-Tito, T. Robledo, E. Perez Salazar; Cell Biology, Cinvestav-IPN, Mexico DF, Mexico

Breast cancer is one of the major public health problems among women worldwide. A number of epidemiological studies have been carried out to elucidate the role of dietary fat and the risk of breast cancer. Recently, it has been reported that two G protein-coupled receptors (GPR40 and GPR120) are activated by medium- and long-chain fatty acids, such as oleic acid (OA), the predominant free fatty acid in blood circulation and diet. Previous reports and data from our laboratory show that these receptors are expressed in breast cancer cell lines, and OA promotes proliferation. However, the signal transduction pathways mediated by OA, remain to be investigated. In the present study, our aim was to study the signal transduction pathways that participate in the proliferation induced by OA. By using DNA synthesis assays, Western

blot, EMSA, and inhibitors of Src, EGFR, MMPs, and ERK1/2, we demonstrated that OA induces activation of GPCRs, Src, EGFR, MMPs and AP-1 DNA complex formation. In addition, OA promotes proliferation in a fashion dependent of ERK1/2 and EGFR transactivation.

1967/B430

Altered Metabolism and Signaling in Mouse Embryonic Fibroblasts Derived from an O-Glcnacase (OGA) Knockout.

C. Keembiyehetty[1], M. Comly[1], D. Love[1], G. W. Robinson[2], L. Hennighausen[2], J. Hanover[1]; [1]Lab of Cell Biochemistry and Biology, NIH /NIDDK, Bethesda, MD, [2]lab of genetics and Physiology, NIH /NIDDK, Bethesda, MD

O- GlcNAc Transferase (OGT) and O-GlcNAcase (OGA) are enzymes that modify proteins by adding and cleaving a single O-GlcNAc moiety respectively. The O-GlcNAc modification occurs at serine and/or threonine residues and is analogous to phosphorylation. Perturbation of O-GlcNAc cycling is thought to be associated with diabetes, Alzheimer's disease and cancer. In humans, the OGA gene is a documented diabetes susceptibility locus associated with high incidents of diabetes among Mexican Americans. In this study, we generated an OGA knockout mouse model to investigate the role of the O-GlcNAc modification in diabetes and cellular signaling. An OGA floxed construct was designed by inserting a neomycin cassette flanked by lox P sequences between exon1 and 2, with a third lox P site in front of exon1. OGA chimera mice bearing heterozygous floxed allele were derived and crossed. The homozygous floxed allele did not produce viable offspring. However, we were able to obtain mouse embryonic fibroblasts (MEFs) with a homozygous floxed allele. Heterozygous floxed mice were bred with cre recombinase expressing mice to obtain a heterozygous knockout allele of OGA. The homozygous OGA KO mice were embryonic lethal. Therefore, MEFs were collected at embryonic day 12.5 to obtain the homozygous OGA KO MEFs. We confirmed that all OGA isoforms were ablated in the OGA KO cell lines. O-GlcNAcylated protein levels were increased in OGAKO cells compared to wild type. Semi quantitative RT-PCR studies indicated that expression levels of several metabolic genes were altered in OGA deleted cells including IRS1, AMPK and GSK 3 beta. These findings suggest that loss of mammalian OGA leads to embryonic lethality and alters cellular signaling cascades consistent with the proposed role of O-GlcNAcase in the pathophysiology of type 2 diabetes mellitus.

1968/B431

Epigenetic Regulation of Human Hedgehog Interacting Protein (HHIP) Gene in Medulloblastoma, Glioblastoma and Neuroblastoma.

M. H. Shahi[1,2,3], M. Afzal[1], J. Castresana[1,3]; [1]Aligarh Muslim University, Aligarh, India, [2]All India Institute of Medical Sciences(AIIMS), New Delhi, India, [3]Universidad de Navarra, Pamplona, Spain

Objective: The Human Hedgehog Interacting Protein (HHIP), a putative antagonist of the hedgehog signaling has been reported to be methylated in a variety of tumors such as gastric, pancreatic, colorectal, esophageal and lung cancer. The sonic hedgehog (SHH) pathway is well determined in medulloblastoma and under much investigation in glioblastoma and neuroblastoma development. In this study we have determined the expression of HHIP and its epigenetic status in medulloblastoma, glioblastoma and neuroblastoma cell lines and samples. Methods: We have assessed HHIP transcript expression by qRT-PCR in medulloblastoma, glioblastoma and neuroblastoma cell lines and samples. Then we have done promoter methylation analysis of HHIP by MCA-Meth (melting curve analysis). We have also treated the cell lines with the demethylating agent 5-Aza-2'-deoxycytidine and the HDAC inhibitor TSA. Results: We found all 6 medulloblastoma cell lines, 3/14 (21%) medulloblastoma samples, 6/8 (76%) glioblastoma cell lines, 34/43 (79%) primary glioblastoma tumor samples and 2/11 (18%) neuroblastoma cell lines showed methylation/partial methylation in the HHIP promoter region by MCA-Meth. Expression of the HHIP transcript was restored in these unexpressed tumor cell lines after treatments with 5-Aza-2'-deoxycytidine and TSA. Conclusion: Low expression of HHIP transcript was determined in cell lines and samples of these three tumors. The HHIP gene is epigenetically controlled. This low expression of HHIP might prompt upregulation of the SHH pathway in these three major tumors of the nervous system.

1969/B432

Understanding the Assembly of TOR Complexes 1 and 2.

A. W. Hill, T. Powers; Molecular and Cellular Biology, UC Davis, Davis, CA

The TOR (target of rapamycin) proteins are large, highly conserved, serine/threonine protein kinases that assemble into two distinct complexes, termed TOR Complex 1 and 2 (TORC1 and TORC2), which regulate both temporal and spatial aspects of cell growth in eukaryotic cells. In *S. cerevisiae*, there are two highly similar TOR proteins, Tor1 and Tor2. Tor1 can assemble only into TORC1, but Tor2 can assemble into TORC1 and TORC2. Only Tor2 is essential in yeast. It has been demonstrated previously that the N-terminal half of Tor2, consisting primarily of coil-coil repeats termed the HEAT repeats region and the FAT domain, is responsible for the ability of Tor2 to function in both complexes. To address the question of this region's importance in complex-specificity, we have created chimeric TOR genes that contain specific sequences of TOR2 within the context of an otherwise TOR1 gene, to assess the importance of different regions within the HEAT and FAT domains. Using this approach we have identified a region of 522 amino acids in length, located within the more N-terminal portion of Tor2, that confers significant TORC2 behavior, including stable biochemical interactions, detected by co-immunoprecipitation, with known TORC2-specific binding partners as well as selective recognition of a TORC2-specific substrate, the AGC kinase Ypk2, detected by *in vitro* kinase assay. Interestingly, modeling studies indicate that this region is likely to fold as an independent structural domain, which could account for its behavior in these studies. Remarkably, we have also identified a number of independent and distinct segments of Tor2, between 300 and 600 amino acids long, which can independently confer some TORC2 behavior, based on a given chimeras' ability to functionally replace Tor2 *In Vivo* in mutants lacking a genomic copy of TOR2. However, biochemical examination of these chimeras revealed that they maintain stable interactions with TORC1 components and are inefficient at recognizing Ypk2. We conclude that there are likely to be multiple assembly determinants for TORC2, distributed throughout the HEAT repeats region of Tor2, but that the more N-terminal repeats dominate in terms of stable assembly into TORC2.

1970/B433

Status of the TOR Signaling Pathway in Cytokinesis Mutant Strains of the Budding Yeast Saccharomyces Cerevisiae.

G. Pagan-Mercado, J. Rodriguez-Medina; Biochemistry, School of Medicine, Medical Sciences Campus, University of Puerto Rico, San Juan, PR

The *Saccharomyces cerevisiae* TOR kinases, *TOR1* and *TOR2*, share the essential signaling functions of controlling translation initiation and cell growth in response to availability of nutrients. Inactivation of the TOR pathway dissociates Sit4 phosphatase from Tap42p, resulting in down-regulation of translational initiation, repression of ribosome biogenesis, cell cycle arrest, induction of autophagy, and acquisition of thermotolerance. A downregulation of ribosomal protein (RP) genes was observed for *myo1Δ* and *chs2Δ* cytokinesis mutant strains, which suggested that the TOR pathway may be inactivated in these strains. The activity of this pathway can be assayed by analyzing the phosphorylation state of its downstream effectors Npr1p and Tip41p. Npr1p and Tip41p are inactive when phosphorylated and become active in the dephosphorylated state. The objective of this study was to determine whether the TOR pathway was inactivated in our cytokinesis mutant strains. In order to elucidate the status of the pathway in mutant strains *myo1Δ*, *chs2Δ* and a control cell wall mutant *fks1Δ*, these were transformed with plasmid pEJ23 containing N-terminally tagged HA-*NPR1* (kindly provided by Dr. Estela Jacinto). Western Blot was used to determine the phosphorylation state of the tagged protein. Our preliminary results show that HA-*NPR1* was phosphorylated in our mutant strains. In addition, there were differences in the level of expression of the recombinant Npr1p. Our preliminary conclusion is that the absence of a change in the level of phosphorylation of Npr1p in our mutants suggests that the TOR pathway maintains normal activity and the transcriptional repression of RP genes may occur through a TOR-independent pathway. [This work was supported by MBRS-SCORE (1SC1AI081658-01), with partial support from NCRR-RCMI (G12RR-03051)].

1971/B434

Novel Transcriptional Variants of Human Serum/Glucocorticoid Regulated Kinase 1 Show Differential Localisation and Regulation.

T. R. Jackson[1], B. Hall[1,2], N. A. Daniels[1], D. Jamieson[1], N. J. Reynolds[1], Q. Long[1], S. Conzen[2]; [1]Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, United Kingdom, [2]Department of Medicine, University of Chicago, Chicago, IL

Serum/glucocorticoid regulated kinase1 (SGK1) is a ubiquitous kinase that regulates homeostatic, cell cycle progression and cell survival responses. SGK1 levels are known to be regulated at transcriptional, translational and postranslational levels by hormones, cytokines and stress stimuli. Like the closely related Akt kinases activation of SGK1 is Phosphoinositide 3-Kinase (PI 3-K) regulated and requires phosphorylation of T-loop and C-terminal hydrophobic motif residues by PDK1 and mTOR kinases respectively. Investigation of the role of SGK1 in human keratinocytes exposed to serum or oxidative stress led us to identify novel SGK1 isoforms activated by each. We identified 5 different transcripts encoding variants we term SGK1A,B,C,Dand F. These arise by differential use of alternative splicing and transcriptional start sites extending the size of the SGK1 locus on chomosome 6q23 to more than 150kbp. We found all variants widely expressed in normal human tissues but relative levels of C and D varied between immortalised and normal keratinocytes. Each variant encodes a unique amino-terminus and on ectopic expression generated a distinct product of appropriate predicted mass. Expression showed distinct subcellular distributions from specific association with mitochondria (A), mitochondrial and diffuse (B&D) to diffuse nuclear and cytoplasmic localisation alone (C&F). Additionally the 60kDa SGK1D variant showed a unique PI 3-K-dependent plasma membrane-association. Plasma membrane association was abolished by mutation of a conserved Arginine in a potential PX domain unique to this isoform. Further phosphospecific-antisera indicated that hydrogen peroxide mediated phosphorylation of the 60kDa SGK1 variant is uniquely PI 3-K-dependent. Our findings raise exciting questions regarding the identity of the variants regulated by differing hormonal, growth and stress stimuli. Furthermore we conclude that these transcriptional variants allow differential localisation and regulation of SGK1 providing a mechanism for this kinase to participate in multiple discrete signalling pathways controlling cell physiology, growth and survival.

Calcium and Calcium Binding Proteins (1972 – 1981)

1972/B435

Calcium Influx Channels in Breast Cancer: Altered Expression of TRPV6 and TRPV1 in a Subset of Breast Cancer Cell Lines.

D. McAndrew, S. J. Roberts-Thomson, G. R. Monteith; School of Pharmacy, University of Queensland, Brisbane, QLD, Australia

Ionized calcium (Ca^{2+}) is essential for the survival of all eukaryotic cells. Because Ca^{2+} is an indispensable component in a profusion of signaling pathways, its concentration and location within the cell must be tightly regulated. Alterations in Ca^{2+} homeostasis can compromise the integrity of signaling pathways, including those responsible for proliferation, differentiation, and cell death. Alterations in channel expression are a potential cause of errant Ca^{2+} signaling, a process that may occur during carcinogenesis. Alterations in the expression of various Ca^{2+} transporters occurs in breast cancer cells, but many Ca^{2+} channels have not been widely investigated in breast cancer. Our aim was to investigate whether the expression of specific channels expressed in the mammary gland is altered in breast cancer cells. We used real time RT-PCR to examine the expression of specific calcium channels (many members of the TRP superfamily) in two normal breast epithelial cell lines and in six breast cancer cell lines. We identified Ca^{2+} channels with altered expression in breast cancer cell lines. For example, TRPV1 expression was higher in all breast cancer cell lines compared to the normal breast epithelial cell lines, with a greater than 10-fold increase in one cancer cell line (T-47D). TRPV6 was increased (>100-fold) in three breast cancer cell lines (T-47D, ZR-75-1, and SK-BR-3), relative to normal breast epithelial cell lines. Alterations in channel expression were independent of estrogen receptor expression. The functional consequences of inhibiting Ca^{2+} channel expression using siRNA on calcium signaling and proliferation has also been assessed. These results suggest that alterations in the expression of specific Ca^{2+} channels can be a characterizing feature of some breast cancer cells and some may be targets in specific breast cancer cell types.

1973/B436

Auroraa and HEF1 Regulate Polycystin 2.

O. V. Plotnikova[1], E. Pugacheva[2], E. Golemis[1]; [1]Division of Basic Science, Fox Chase Cancer Center, Philadelphia, PA, [2]Department of Biochemistry, Mary Babb Randolph Cancer Center; and West Virginia University, School of Medicine, Morgantown, WV

Polycystic kidney disease (PKD) is common hereditary kidney disease characterized by progressive cyst formation and ultimate loss of renal function. Genetically, PKD results from mutation in either the PKD1 or PKD2 genes, which encode the proteins polycystin 1 and 2 (PC1 and PC2) that heterodimerize in the cilia of cells within kidney tubules. Our laboratory has described an interaction between HEF1, a multidomain scaffolding protein, and the Aurora-A (AurA) kinase at primary cilia that controls ciliary disassembly. Defective ciliary signaling leads to renal cell non-responsiveness to changes in urine flow which would normally increase the level of calcium (Ca2+) within cells. This abnormality in Ca2+ signaling trigger cells to grow abnormally, and ultimately contributes to cyst formation. HEF1 and AurA are both abundantly expressed in human and murine kidneys: we wished to establish if there were physical or functional relationships involving Aurora-A, HEF1, and PKD proteins. We have found that AurA phosphorylates PC2 in vitro at a single site, S829, a high scoring AurA consensus phosphorylation motif. Mutation of this site abolishes AurA phosphorylation of PC2. We have demonstrated that AurA and HEF1 coimmunoprecipitate with overexpressed or endogenous PC2 in mammalian cells, and that HEF1 promotes AurA phosphorylation of the PC2 C-terminus in vitro. Importantly, AurA phosphorylation negatively regulates Ca2+-dependent activation of the PC2 Ca2+ channel activity, limiting magnitude and duration of Ca2+ release. Small molecule inhibitors of AurA such as PHA-680632 increase PC2 activity, while S829A-mutated PC2 is resistant to inhibition of AurA. Overall, the current data support the ideas that HEF1 and AurA are novel regulators of PC2 activity, and that inhibition of AurA may have therapeutic benefit in PKD associated with PKD1 mutations.

1974/B437

Bordetella Pertussis CyaA-RTX Subdomain Requires Calcium for Structural Stabilisation.

P. Pojanapotha[1], B. Powthongchin[2], C. Angsuthanasombat[1]; [1]Institute of Molecular Biology and Genetics, Mahidol University, Nakorn Pathom, Thailand, [2]Department of Biopharmacy, Faculty of Pharmacy, Silpakorn University, Nakorn Pathom, Thailand

The Bordetella pertussis adenylate cyclase-haemolysin toxin (CyaA) is a calcium-binding protein in the repeat-in-toxin (RTX) family which contain the GGXGXDXUX repetitive sequence as a special characteristic. The RTX subdomain located at the C-terminus serves as a calcium-binding portion. CyaA is distinctive among the other RTX toxins since it contains the highest number of repeats (~40) in the region while most of the RTX members consist of only 7-13 repeats. In this study, we have cloned the RTX subdomain of CyaA (residues 751-1706) for structural and functional characterisation. The 100-kDa protein was overexpressed as a soluble form in Escherichia coli under control of the T7 promotor. The protein purified using anion-exchange chromatography was found to be highly sensitive to proteolytic degradation. By trying with various types of protease inhibitors, PMSF (serine protease inhibitor) and EDTA gave the best result among the others, albeit incomplete inhibition. However, the purified RTX subdomain with enhanced stability to protease degradation was obtained by adding CaCl2 in millimolar range (5-10 mM) to the preparation buffers in the absence of inhibitors, suggesting that calcium plays an important role in stabilising a native folded conformation of the 100-kDa RTX subdomain of CyaA. Part of the CyaA-RTX subdomain was modelled based on the crystal structure of the Pseudomonas aeruginosa alkaline protease, a member of the RTX family. The model reveals that calcium ions are located between the loops linking beta sheets together, suggesting that the ions act as a bridge to compact the protein into a stable conformation. Altogether, this report demonstrates for the first time that structural stabilisation of the CyaA-RTX subdomain is calcium dependent.

1975/B438

Role of Nicotinic Acetylcholine Receptor on Intracellular Calcium Signaling Mediated by Cellular Prion Protein.

F. Beraldo[1], C. Arantes[1,2], C. Machado[1,3], R. Linden[4], M. A. Prado[5], R. Markus[6], V. R. Martins[1]; [1]BCM, Ludwig Institute for Cancer Research, Sao Paulo, Brazil, [2]Biochemistry, São Paulo University, São Paulo, Brazil, [3]RTC, Cancer Hospital AC Camargo, São Paulo, Brazil, [4]Biophysical, Rio de Janeiro University, Rio de Janeiro, Brazil, [5]Pharmacology, Minas Gerais University, Belo Horizonte, Brazil, [6]Physiology, São Paulo University, São Paulo, Brazil

The cellular prion protein (PrPC) is a highly conserved cell surface glycoprotein expressed in the Central Nervous System. Its conformationally modified isoform is associated with Transmissible Spongiform Encephalopathies or prion diseases. Recently, multiple and diverse functions of PrPC have been reported. Stress Inducible Protein 1 (STI1) is a PrPC ligand, which promotes neuronal differentiation and neuroprotection via Extracellular signal-regulated kinase 1 and 2 (ERK1/2) and cAMP-dependent Protein Kinase 1 (PKA) phosphorylation (Lopes et al., J. Neurosc. 2005 Dec 7;25(49):11330-9.). Using an intracellular Ca2+ probe, Fluo- 3 AM, we evaluated the STI1 effect on Ca2+ signaling. In hippocampal neurons, STI1-PrPC interaction induced an increase in intracellular Ca2+ levels through extracellular Ca2+ influx. Conversely, there was no effect on intracellular calcium when neurons were treated with a STI1 which lacks the PrPC binding domain (STI1Δ230-245) or when PrPC-null cells were treated with STI1. Moreover, PrPC-STI1 interaction was also able to induce ERK1/2 phosphorylation and PKA activation in a Ca2+ influx-dependent matter. These data indicate that calcium influx mediated by STI-PrPC binding is upstream to ERK1/2 and PKA activation, and the former is promoted by a calcium channel at the plasma membrane. Inhibitors for several types of calcium channel such as store operated channels, voltage gated channels and ligand-gated ion channels (SKF and nifedipine, respectively) had no effect on STI1-induced calcium signaling. Remarkable, α-bungarotoxin, which is a specific inhibitor for the ligand-gated channel type nicotinic acetylcholine receptor, was able to completely block Ca2+ increase as well ERK1/2 and/or PKA activation mediated by STI1-PrPC interaction. Thus, our results indicate that PrPC binding to STI1 modulates nicotinic acetylcholine receptor promoting Ca2+ influx and activation of ERK1/2 and PKA. Supported by - FAPESP and HHMI

1976/B439

Microtubule Remodeling Mediates the Inhibition of Store-Operated Calcium Entry (SOCE) during Mitosis in COS-7 Cells.

R. A. Denis, T. Saino, H. Akutsu, Y. Satoh; Anatomy (Cell Biology), Iwate Medical University, Morioka, Japan

[Objective] Calcium signaling controls diverse and vital cellular processes, and regulation of the intracellular calcium ion concentration ([Ca2+]i) is critical. However, there is limited evidence as to whether differences of [Ca2+]i dynamics exists among various cell cycle stages. Here we will examine and compare the [Ca2+]i dynamics of COS-7 cells in mitotic and non-mitotic phases with special reference to store-operated calcium entry (SOCE), which is the main mechanism through which many cells maintain the delicate balance. **[Methods]** Using laser scanning confocal microscopy (Zeiss 510) to monitor [Ca2+]i dynamics (Fluo-4 was used as a [Ca2+]i indicator), and to show

cytoskeletons. Cells were perfused with Hepes Ringer buffer (HR) containing or lacking extracellular Ca2+. To elicit SOCE, thapsigargin or ATP were used. **[Results]** During thapsigargin or ATP treatment with extracellular Ca2+-free HR, [Ca2+]i inceased transiently, because of Ca2+ mobilization from internal Ca2+ store. Thereafter when cells were perfused with HR containing extracellular Ca2+, SOCE was induced in non-mitotic cells, but considerably inhibited in mitosis, and virtually absent during the metaphase. Various cytoskeletal modifying drugs were used to assess the contribution of microtubule and actin filaments in SOCE signaling. Nocodazole (to interfere the polymerization of microtubules) caused microtubule network disorganization; in many cases, the tubules retracted from cell periphery that mimicked the natural mitotic microtubule remodeling. After the nocodazole treatment, cells showing distinct microtubule disorganization, SOCE was inhibited. In accordance with it, endoplasmic reticulum (a candidate of internal Ca2+ store) dislocated from periphery to center of the cell. Short exposure to paclitaxel (inhibition of microtubule depolumerization, and over-stabilizing microtubule network) bolstered SOCE, whereas long exposure resulted in microtubule disruption and SOCE inhibition. Cytochalasin D and calyculin A (actin-modifying drugs) did not affect SOCE. **[Conclusion]** Mitotic microtubule remodeling, but not actin filament remodeling, contributes inhibition of SOCE in Mitotic phase [Ca2+]i dynamics.

1977/B440

Expression of Chromogranins A and B, and Secretogranin II in Human Glial Cells Oligodendrocytes and Astrocytes.

K. D. Kim, Y. S. Huh, S. Yoo; Biochemistry, Inha University School of Medicine, Incheon, South Korea

Chromogranins A and B, and secretogranin II, three major members of the granin family proteins that are traditionally known as secretory granule marker proteins, are high capacity, low affinity Ca2+ storage proteins that also activate the IP3R/Ca2+ channels. The presence of chromogranin A in glial astrocytes has been reported before, but the presence of chromogranin B and secretogranin II is not studied well. Moreover, the presence of these proteins in glial oligodendrocytes has not been studied before. Therefore, we investigated the localization of chromogranins A and B, and secretogranin II in human glial cells using immunogold electron microscopy, and found the presence of chromogranins and secretogranin II in glial cells astrocytes and oligodendrocytes. All three proteins were localized in dense core vesicles (secretory granules) that are present in the cytoplasm of these cells, but chromogranin B and secretogranin II were also present inside the nucleus. The presence of dense core vesicles (secretory granules) in glial astrocytes and oligodendrocytes suggests that these glial cells participate in some form of secretory function in the brain, implying potentially important roles of these glial cells in signal transduction or cell-to-cell communication in the brain.

1978/B441

NAADP and Cadpr Regulate Intracellular Ca2+ Level in IL8-Induced Migration of Lymphokine-Activated Killer Cells.

T. Nam, S. Rah, U. Kim; Biochemistry, Chonbuk National University Medical School, Jeonju, South Korea

We have previously demonstrated that a calcium signaling messenger, cyclic ADP-ribose (cADPR), is formed via inositol 1,4,5-trisphosphate (IP_3)-mediated Ca^{2+} increase in interleukin 8 (IL8)-induced lymphokine-activated killer (LAK) cells (Rah et al, 2005). This study was performed to test whether another Ca^{2+} signaling messenger, nicotinic acid adenine dinucleotide phosphate (NAADP) is generated in the IL8-treated LAK cells. IL8 induced to form NAADP following cADPR production by CD38 activation, as LAK cells prepared from $CD38^{-/-}$ mice did not produced the messengers by the treatment with IL8. IP_3 receptor blocker, xestospongin C completely blocked the formations of cADPR and NAADP as well as IL8-induced Ca^{2+} signal. Exogenous cADPR application could induce an increase of intracellular NAADP level whereas NAADP did not increase intracellular cADPR level. Moreover, the treatment of LAK cells from $CD38^{-/-}$ mice with cADPR did not induced NAADP formation and the cell migration. However, NAADP increased intracellular Ca^{2+} signal and cell migration in the LAK cells either from wild type or from $CD38^{-/-}$ mice, which were completely blocked by bafilomycin A1, an inhibitor of the lysosomal H^+-ATPase. Consistent with our previous finding of cGMP-dependent cADPR production, cGMP analog could induce formations of both cADPR and NAADP. In contrast, cAMP analog induced to only NAADP production. Taken together, our data suggest that the two Ca^{2+} signaling messengers, cADPR and NAADP are spatiotemporally or differentially produced by CD38. Supported by KOSEF grant R0A-2007-000-20121-0.

1979/B442

Functional and Structural Studies of Secretagogin, a Novel Calcium Binding Protein in Insulinoma, Using Proteomic Analysis.

H. Lee, J. Jeong, J. Lee, D. Shin, K. Lee; College of Pharmacy and Division of Life & Pharmaceutical Sciences, The Center for Cell Signaling and Drug Discovery Research, Ewha Womans University, seoul, South Korea

Secretagogin (SCGN) is a novel hexa-EF-hand calcium binding protein. SCGN is highly expressed in pancreatic β-cell and neuroendocrine cells, especially in insulinoma cells. Although the biological function of SCGN is suggested to be involved in insulin secretion, the molecular mechanism of this functional role in vivo is not exactly understood. In this study, we identified that 4 out of 6 EF-hands have high calcium binding affinity bioinformatic analysis. It is known that three dimensional structures of calcium binding proteins are changed depending on calcium concentrations and regulated the protein-protein interactions and cellular functions. We performed the functional and structural studies employing proteomic analysis. First, the interacting proteins of SCGN were identified in calcium dependent manner in HEK293T cells combining immunoprecipitation and nanoLC-ESI-q-TOF tandem MS. Secondly, the structural changes of SCGN were investigated by calcium binding. Thirdly, multiple post-translational modifications of SCGN including disulfide bond depending on calcium concentration were identified employing nanoLC-ESI-q-TOF tandem MS. The results suggested that a novel calcium binding protein SCGN can induce post-translational modifications, alter three dimensional structures, and regulate the interacting proteins and its biological functions depending on calcium concentration. [Supported by KOSEF NCRC for CCS & DDR. H Lee and J Lee supported by BK21]

1980/B443

Identification of the Protein Targets of CpnA in Dictyostelium.

H. Mao[1], L. K. Ho[2], M. Bayeva[2], L. C. Naliboff[2], E. S. Eberhardt[4], D. Loiselle[3], T. A. Haystead[3], C. K. Damer[1]; [1]Biology, Central Michigan

University, Mount Pleasant, MI, [2]Biology, Vassar College, Poughkeepsie, NY, [3]Pharmacology and Cancer Biology, Duke University Medical Center, Durham, NC, [4]Chemistry, Vassar College, Poughkeepsie, NY

Copines make up a family of calcium-dependent membrane-binding proteins found in a diverse array of eukaryotic organisms. Copines are characterized by having two C2 domains in the N-terminal half of the protein followed by a VWA domain in the C-terminal half. A general hypothesis for the function of copines is to act in signaling pathways as chaperone proteins that bring target proteins to membranes in response to an increase in the intracellular calcium concentration. We are studying copines in the model organism, Dictyostelium discoideum, and are using affinity chromatography and immunoprecipitation techniques to isolate the target proteins of one of the copine proteins, CpnA. A green fluorescence protein (GFP)-tagged version of CpnA was expressed in Dictyostelium, purified, and linked to a column. Dictyostelium cell lysates were then run over the column and eluted first with EGTA, then with low pH. Six proteins found in the eluates of the CpnA-linked column and not the eluates of the control column were identified by peptide mass fingerprinting with mass spectrometry: discoidin I and II, actin, acetylornithine deacetylase, and S-adenosyl-L-homocysteine hydrolase. Discoidins are proteins only found in Dictyostelium and are often found to contaminate column fractions. However, discoidin-like domains are found in several mammalian proteins and have been shown to interact with proteins with VWA domains suggesting that the discoidins may be true targets of CpnA. To corroborate these findings and identify other targets of CpnA, we performed immunoprecipitations using a polyclonal antibody to GFP with Dictyostelium cells expressing GFP, GFP-CpnA, or GFP-fused to the VWA domain of CpnA. The GFP antibody is able to pull-down GFP and the GFP-fusion proteins. Protein gel analysis indicates that there are proteins that co-immunoprecipitate with the GFP-fusion proteins and not with GFP. These candidate target proteins will also be identified by peptide mass fingerprinting using mass spectrometry.

1981/B444

Association Analysis of PLCG1 (Phospholipase C, Gamma1) with Hepatocellular Carcinoma in a Korean Population.

A. Oh, S. Lee, H. Kang, K. Kim, J. Kim, J. Pyun, S. Won, K. Lee, H. Song, K. Kwack; CHA Research Institute, Pochon CHA Univ., Seongnam, South Korea

Phospholipase C, gamma 1 (PLCG1), as an important component of signal transduction processes, involved in several cellular processes including mitogenesis, differentiation, transformation, and regulate tumor migration. We have examined the relationship between polymorphism of the PLCG1 gene and the risk of hepatocellular carcinoma (HCC) in patients with chronic hepatitis B virus (HBV) infection. A total of 314 Korean subjects were prospectively enrolled; 96 patients with HCC and 218 patients with chronic liver diseases (CLD). Six single nucleotide polymorphisms (SNPs) in PLCG1 gene were genotyped using by the Golden gate genotyping assay. Out of six SNPs, only one was polymorphic, while the remaining five were monomorphic. This SNP is non-synonymous SNP on RT loop in SH3 domain of PLCG1. G/G genotype was significantly associated with an increased risk of HCC. In this study, we provide additional evidence of an association of the PLCG1 polymorphism with HCC as markers of genetic susceptibility in a Korean population.

Apoptosis III (1982 – 1999)

1982/B445

The Mechanism of DZA-Induced Cytochrome C Release in HL-60 Cells.

S. Lee, K. Ko, S. Jeong, H. Kim; Department of Biochemistry, College of Medicine The Catholic University of Korea, Seoul, South Korea

3-Deazaadenosine (DZA), an inhibitor of S-adenosylhomocysteine hydrolase, was reported to induce apoptosis of human leukemic cells dependent on cytochrome c release and caspase activation. But the mechanism involved in the release of cytochrome c by DZA was not clarified yet. In this study we tried to understand the mechanism of DZA-induced cytochrome c release in HL-60 (human promyelocytic leukemia) cells. DZA induced apoptosis in HL-60 cells which was completely blocked by z-VAD-Fmk, a pancaspase inhibitor. Concomitantly with the induction of apoptosis, cytochrome c was released from mitochondria, which was not prevented by z-VAD-Fmk and not associated with dissipation of mitochondrial membrane potential, either. In addition, even when purified mitochondria were incubated with DZA, cytochrome c was not released from mitochondria. However, cytosolic fractions of DZA-treated cells induced the release of cytochome c from purified mitochondria. Put together these findings, it can be suggested that DZA induces the activation of (a) cytosolic protein(s) which stimulate(s) outer mitochondrial membranes to release cytochrome c in HL-60 cells in a caspase-independent manner.

1983/B446

Negative Regulation of the Metabolic Branch of Insulin Signaling on IRS Proteins via Intracellular Glycosylation.

C. Teo, E. G. El-Karim, L. Wells; BCMB, Complex Carbohydrate Research Center, Athens, GA

O-linked beta-N-acetyl-D-glucosamine (O-GlcNAc) modification is a ubiquitous intracellular glycosylation found on serine or threonine residues of nuclear and cytoplasmic proteins. In conjunction with phosphorylation, O-GlcNAc cycling participates in a variety of cellular processes. Notably, it acts as a sensor for glucose flux through the hexosamine biosynthetic pathway and has been tightly linked to the development of insulin resistance, which precedes and is a hallmark for type II diabetes, in both cell culture and animal models. In this study, we dissected the impact of O-GlcNAc modification on the metabolic branch of the insulin signaling pathway. By using mammalian cell culture models, we report that elevated O-GlcNAc levels via pharmacological and genetic manipulations inhibit the anti-apoptotic action of insulin by negatively modulating the insulin-mediated signaling cascade with a concomitant defect at or upstream of Akt activation. Also, many of the proteins involved in the pathway are found to be O-GlcNAc modified. Since activation of the PI3K/Akt pathway is conserved in different receptor tyrosine kinase (RTK)-mediated signaling pathways, we investigated the activation status of the PI3K/Akt cassette via two other predominant RTK pathways mediated by epidermal growth factor (EGF) and platelet-derived growth factor (PDGF) in response to global increases in O-GlcNAc levels. Our results clearly demonstrate that attenuation of Akt activation in response to elevated O-GlcNAc levels is exclusively an insulin signaling-specific phenomenon. Given that increasing O-GlcNAc levels does not affect the autophosphorylation activity of the insulin receptor, our results strongly suggest that the IRS proteins, which are O-GlcNAc modified, are the

target for functional O-GlcNAc modification and fine-tuning of canonical insulin-mediated signal transduction. This work was supported by an American Heart Association predoctoral fellowship (CFT) and NIDDK/NIH 1R01DK075069 (LW).

1984/B447

Modulation of Heme Oxygenase Activity Regulates the Expression of P53 in Human RPE Cells.

K. Kim, S. Park, J. Song, Y. Park; Microbiology and Immunology, Pusan National University School of Medicine, Busan, South Korea

Heme oxygenase (HO-1) is a stress-responsive protein that is known to regulate cellular functions such as cell proliferation, inflammation, and apoptosis. Here, we investigated the effects of CoPP and ZnPP, an activator and an inhibitor of HO, respectively, on the expression of p53 in human retinal pigment epithelium (RPE) cells, along with the possible relationships among these factors. Cobalt protoporphyrin (CoPP) induced the expression of both HO-1 and p53 without significant toxicity to the cells. In addition, the blockage of HO activity with the iron chelator DFO or with HO-1 siRNA inhibited the CoPP-induced expression of p53. Similarly, zinc protoporphyrin (ZnPP), an inhibitor of HO, suppressed p53 expression in ARPE-19 cells, although ZnPP increased the level of HO-1 protein while inhibiting HO activity. Also, CoPP-induced p53 expression was not affected by the formation of reactive oxygen species (ROS). Based on these results, we conclude that HO activity is involved in the regulation of p53 expression in a ROS-independent mechanism, and also suggest that the expression of p53 in ARPE-19 cells is associated with heme metabolites such as biliverdin/bilirubin, carbon monoxide, and iron produced by the activity of HO.

1985/B448

Diphenyleneiodonium, a Flavoenzyme Inhibitor, Induces ROS-Independent P53 Expression and Apoptosis in Human RPE Cells.

S. Park, K. Kim, J. Song, Y. Park; Microbiology and Immunology, Pusan National University School of Medicine, Busan, South Korea

Diphenyleneiodonium (DPI) is a well-known inhibitor of flavoenzymes. It has been reported to inhibit the activity of NADPH oxidase, nitric oxide synthase, xanthine oxidase, and NADPH cytochrome P450 oxidoreductase. Despite its nonspecific mode of action, DPI has frequently been used to inhibit reactive oxygen species (ROS) production mediated by flavoenzymes in various types of cells, where the resultant oxidants are proposed to play a role in cell signaling. In this study, we investigated the effect of DPI on the apoptosis of human RPE cells. DPI treatment in ARPE-19 cells evoked a dose- and time-dependent growth inhibition, and also induced DNA fragmentation and protein content of the proapoptotic factor Bax. In addition, DPI significantly induced the expression and phosphorylation of p53, which induces proapoptotic genes in response to DNA damage or irreparable cell cycle arrest. ROS have been implicated as a key factor in the activation of p53 by many chemotherapeutic drugs. Recent data on the regulation of intracellular ROS by DPI are controversial. Therefore, we analyzed whether DPI could contribute to the generation of intracellular ROS. Although there was increase in ROS level from cells treated for 24 h with DPI, it was not detectable at early time points, required to induce p53 expression. And DPI-induced p53 expression was not affected by the ROS scavenger NAC. We conclude that DPI induces the expression of p53 by ROS-independent mechanism in ARPE-19 cells, and renders cells sensitive to drug-induced apoptosis by induction of p53 expression.

1986/B449

The Role of Retinoblastomabinding Protein 6 (RBBP6) in Cancer Development and Apoptosis.

L. R. Motadi; School of MCB, University of Wits, Johannesburg, South Africa

Introduction RBBP6 is a gene that encodes RBBP6 protein product and has been reported to be involved in mitotic apoptosis and interacts with pRb and as well as p53. This strongly suggests the involvement of this gene in the cell cycle regulation. It has also been reported that this gene is involved in mRNA processing and has ubiquitin ligase activity. The aim was to determine the expression of the RBBP6 gene in lung squamous cell carcinoma. Methods RbBP6 localisation studies were done on paraffin sections, normal and cancer tissues, using in situ and immunocytochemistry. Sterosporine was used for apoptosis induction to see if there was any change of the level of expression in RBBP6 using real time PCR and the ApoPercentange assay was use to check if cells were dying due to apoptosis. Results Both RBBP6 mRNA and protein localization using in situ hybridization and immunocytochemistry showed the accumulation of the RBBP6 gene products in the cytoplasm and very little localization was seen in the nucleus as compared to normal controls. More than 40% of both LC-Sq1, A549 and MRC5 cell line underwent apoptosis when treated with 25nM Steresporine and showed increased expression of RBBP6. These cells died by apoptosis as measured by an ApoPercentage assay. Real-time PCR showed that RBBP6 mRNA expression is increased in cancer cells. Conclusion Cytoplasmic localization of the RBBP6 may suggest that it is translocated to the cytoplasm in order to impede it from performing its apoptotic or cell cycle regulating functions.

1987/B450

Multi-Parametric Analysis of Apoptosis and Necrosis in Cells Expressing a Mitochondria-Targeted Green Fluorescent Protein Construct.

H. Cox, I. Lebedeva, M. Szczepanik, D. Shen, D. Gattica, W. F. Patton; Research and Development, Enzo Life Sciences, Farmingdale, NY

Programmed cell death, or apoptosis, has previously been monitored using a variety of assay methods including DNA laddering on gels, visual assessment of nuclear condensation and fragmentation, annexin V binding, caspase activation and the TUNEL assay. Unfortunately, due to the overall complexity of the apoptotic process, single parameter measurements provide insufficient information, as such assays are typically capable of only providing a binary readout of early or late stage events. A three-parameter cell-based assay for measurement of apoptosis and necrosis is described utilizing a fluorophore conjugate of annexin V for measuring early stage apoptosis, a cell-impermeable fluorescent DNA intercalation dye for measuring late-stage apoptosis/necrosis and a mitochondria-targeted enhanced green fluorescent protein (EGFP) construct for reporting upon loss of mitochondrial membrane potential (MMP) arising from cell injury. HeLa cells expressing EGFP-cytochrome oxidase chimeric protein were exposed to a prototypical apoptosis-inducing agent, staurosporine. After incubation, a DNA-binding dye and cyanine 3E-conjugated annexin V were added to the cells. Time and dose-dependent analysis of the three probes of apoptosis was performed and the temporal order of the underlying processes confirmed. The assay is suitable for rapid and information-rich

analysis of apoptotic events by fluorescence microscopy or flow cytometry. The assay should allow for the rapid screening and identification of novel agents with apoptosis-inducing properties.

1988/B451

Apoptosis and Extrusion: before Death Do Us Part.

D. Andrade, J. Laird, T. Trinh, J. Rosenblatt; Oncological Sciences, University of Utah, Salt Lake City, UT

Epithelia encase tissues and provide a protective barrier, yet the cells of epithelia are constantly undergoing cell division and apoptosis, potentially compromising this barrier. We have found that epithelia maintain their barrier function when their cells undergo apoptosis by a mechanism called cellular extrusion. During extrusion, the dying cell signals its neighboring cells to form and contract an actin and myosin ring that squeezes the apoptotic cell out of the epithelia and simultaneously fills the gap the dying cell might leave, effectively maintaining epithelial integrity. A variety of apoptotic stimuli (Camptothecin, growth factor deprivation and a Fas-stimulating antibody) also induce extrusion in MDCK monolayers, suggesting that the cellular extrusion pathway is conserved regardless of the apoptotic pathway stimulated. Since these pathways activate Bax and induce cytochrome C release, the signal that initiates extrusion likely intersects the mitochondrial pathway for apoptosis. To determine if signals that stimulate extrusion are released from mitochondria upon its permeabilization, we blocked mitochondrial permeabilization with a Bax Inhibitor Peptide (BIP). Treatment of MDCK cells with BIP blocked apoptosis but not extrusion, suggesting that the signal that initiates extrusion might be upstream Bax activation and mitochondrial permeabilization. To test the role of Bcl-2, an anti-apoptotic, Bax-inhibitory protein, in regulating cellular extrusion, we transiently over-expressed Bcl-2 in MDCK cells. Over-expression of Bcl-2 inhibits both apoptosis and extrusion, suggesting that, like Bax, the signal that initiates extrusion might be bound to Bcl-2. HA 14-1, a Bcl-2 inhibitor, is sufficient to induce both apoptosis and extrusion, further supporting this idea. Together, our results suggest that the signal that initiates extrusion is not sequestered in the mitochondria but rather by Bcl-2.

1990/B453

Cannabinoids Inhibit Growth, Migration and Matrix Metalloproteinase 2 in Endothelial Cells.

D. Petit, C. Kramer, P. Bagavandoss; Biological Sciences, Kent State University Stark, Canton, OH

Cannabinoids have been known to not only induce apoptosis in both normal and cancer cells but also inhibit angiogenesis. The objective of the present study was to investigate the effects of cannabinoids on endothelial cell growth, migration and matrix metalloproteinase (MMP) production. All experiments were carried out on EA.hy926 cells, a cell line derived from human umbilical vein endothelial cells, in DMEM:F12 medium in the presence of 5% charcoal-stripped fetal bovine serum. At 15 µM and above, the cannabinoid agonists ACEA (CB1 specific) and JWH 015 (CB2 specific) and antagonists/inverse agonists AM251 (CB1/CB2) and AM630 (CB2) inhibited the growth of these cells in subconfluent cultures during 2-day incubation. At 15 µM the potency of inhibition by these cannabinoids are: AM251 70%, AM630 46%, JWH 015 44%, and ACEA 29%, respectively. The presence of agonists and antagonists together in cultures did not cause any reversal of growth inhibition. In scratch-wound migration assay of confluent monolayer cells, AM251 decreased the migration, whereas AM630 was without effect. However, in the presence of AM630, AM251 further decreased the endothelial cell migration. Analysis of conditioned media showed a marked decrease in MMP-2 and slight increase in MMP-9 in response to AM251. Although AM630 alone did not inhibit the MMP level, in the presence of AM251, it induced further reduction in MMP-2. Our results indicate that cannabinoids, regardless of their receptor specificity, inhibit EA.hy926 endothelial cell growth and migration. The inhibition of migration in the wounded monolayers is also associated with a decrease in MMP-2. (This undergraduate research was supported by funds from the University Research Council and Stark Campus Honors Program).

1991/B454

Yeast BAX-Inhibitor (BXI1) Regulates Apoptosis through the Unfolded Protein Response.

J. Malouin, N. Austriaco; Biology, Providence College, Providence, RI

Bax Inhibitor-1 (BI-1) was first identified by its ability to block Bax-induced apoptotic cell death in the budding yeast, Saccharomyces cerevisiae. Intriguingly, BI-1 is one of only a few cell death inhibitors found in a wide range of fungi, plant, and animal species including S. cerevisiae, D. melanogaster, A. thaliana, M. musculus, and H. sapiens. Recent studies in mammalian and plant cells have suggested that BI-1 isinvolved in the cell's response to endoplasmic reticulum stress (ER-stress). To better understand the function of BI-1, we are characterizing the yeast homolog of BI-1, which we are calling BXI1. We have generated mutant yeast strains lacking BXI1 and have discovered that they are more sensitive to conditions known to induce ER-stress. The Unfolded Protein Response (UPR) is a response to the accumulation of unfolded proteins in the ER that is mediated by the protein, Ire1p. Intriguingly, the overexpression of Ire1p in cells lacking BXI1 enhances the mutant's ability to survive on drugs known to induce ER-stress. Finally, we have discovered that the Δbxi1 mutant has a higher viability in a 2% glucose solution that is known to induce apoptosis. Our data suggests that yeast Bax-inhibitor may regulate apoptosis through the unfolded protein response.

1992/B455

Protective Effect of Panax Ginseng and Acanthopanax Senticosus on Apoptosis Induced by Heat Stress.

K. Yoon[1], K. Kim[1], H. Lee[1], H. Hong[2], B. Lee[1]; [1]Pochon CHA University, Seongnam-Si, Gyeonggi-Do, South Korea, [2]Korea Food Research Institute, Seongnam-Si, Gyeonggi-Do, South Korea

Heat stress induced gene expression and other cellular responses help limit the damage caused by stress and thus facilitate cellular recovery. Cellular damage also triggers apoptotic cell death through several pathways. We evaluated protective effect of Panax ginseng C. A. Meyer (Fresh ginseng) and Acanthopanax senticosus (Siberian ginseng) on apoptosis and a possible signaling pathway involved during apoptosis induced by heat stress. Rats were divided into the following 5 groups: one that was raised at room temperature (CR), one that was subjected to heat stress (HS) for two weeks (37~38°C, 5hr/day), and 3 that were subjected to heat stress conditions and administrated P. ginseng (FD), A. senticosus (GD) or a mixture of P. ginseng and A. senticosus (FGS). We examined the expression level of HSP genes mRNAs and apoptosis pathway related genes mRNAs in rat liver using reverse transcription polymerase chain reaction (RT-PCR) and Real-Time RT-

PCR. The expression levels of the HSPs were higher in the HS rats than in the CR rats. But, the expression levels of the HSPs in rat, which were administered P. ginseng and A. senticosus extracts during heat stress, were lower than those of the group that was subjected to heat stress only. Specially, the expression levels of apoptosis pathways related genes, which are Fas death domain-associated potein (DAXX), Fas (TNFRSF6)-associated via death domain (FADD) and Caspase3 did not change. These results suggest that administration of P. ginseng and A. senticosus may exert protective effects on heat stress without HSP over-expression by blocking or interrupting the heat stress-induced apoptosis mechanism induced by heat stress.

1993/B456

Role of Heavy Metals in Oxidative Stress and Apoptosis in Saccharomyces Cerevisiae.

A. M. Nargund, J. E. Houghton; Biology, Georgia State University, Atlanta, GA

The widespread use of heavy metals, such as copper and cadmium has only served to heighten concerns with their toxicity, as both metals are linked to a number of cellular dysfunctions, neurodegenerative disorders and associated apoptosis, even at low levels of exposure. In using Saccharomyces cerevisiae as a model system to evaluate the toxic nature of these metals we have been able to demonstrate that cadmium, at least, elicits a series of specific oxidative stress responses in the cell, culminating in a heterogeneous apoptotic response. Here we show that low levels (~8 mM) of copper can also cause a heterogeneous apoptotic response within yeast, as determined by mitochondrial perturbation, superoxide generation, induction and activation of the solitary yeast metacaspase, YCA1, and eventually DNA fragmentation. More importantly, perhaps, we also show that while both metals initiate similar oxidative stress responses within the cell, copper and cadmium show distinct pathways leading to the eventual programmed cell death. In cells exposed to cadmium the metacaspase is required to initiate the mitochondrial hyperpolarization that precedes apoptosis, whereas in copper the mitochondrial disruption appears to precede activation of the yeast metacaspase.

1994/B457

Cisplatin Effects on Caspase Expression in the Growing Follicles and Corpora Lutea in the Ovary.

J. Yeh, J. Peresie, B. Kim, A. Abdelkader; Gynecology-Obstetrics, University at Buffalo, Buffalo, NY

Objective: Cisplatin is a chemotherapeutic agent widely used in the treatment of cancers of different organs. Cisplatin produces ovarian damage. We hypothesized that cisplatin increases caspases activity thus accelerating caspase mediated apoptosis. We examined caspase 9, an initiator caspase, and caspase 3, an effector caspase, in the ovaries of cisplatin treated animals. Methods: Three groups of adult female Sprague Dawley rats, saline, 4.5mg/kg cisplatin and 6 mg/kg cisplatin were included. Animals received 2 injections one week apart and euthanized 5 days after last injection. Both ovaries were removed. One ovary was processed for immunohistochemistry and the other ovary was processed for protein analysis by western blot analysis. Caspase 3 and caspase 9 were detected by immunohistochemistry using anti-caspase 3 antibody (Cell Signaling Technology, Inc) and anti-caspase 9 antibody (Cell Signaling Technology, Inc) respectively. Two reviewers using semi-quantitative H scoring method scored sections. Results: Immunohistochemical analysis of caspase 3 expression revealed an increase in caspase 3 levels with increasing cisplatin dose in preantral and antral follicles and corpora lutea. There was no significant difference in caspase 3 levels between saline and cisplatin groups in the other ovarian structures. Immunohistochemical analysis of caspase 9 expression revealed a decrease in caspase 9 with increasing cisplatin dose in preantral and antral follicles and theca cells. Western blot data showed a decrease in cleaved caspase 3, but no changes in caspase 9, with increasing cisplatin doses. Conclusion: Cisplatin may impact the normal ovary, in part, by increasing caspase 3 activity in the growing ovarian follicles. This would then lead to apoptosis in these structures in the cisplatin exposed ovaries.

1995/B458

Systems Biology Approach for Defining Signaling Pathways in Oxidative Stress Induced Cell Injury.

S. Ramachandran[1], S. Peela[1], P. B. Landon[1], S. Subramaniam[2], R. Lal[1]; [1]Medicine, The Univ. of Chicago, Chicago, IL, [2]Bioengineering, Univ. of California at San Diego, La Jolla, CA

Oxidative stress (OS) is a common denominator of many diseases including smoking disorders, atherosclerosis, Parkinson's disease, Alzheimer's disease, ALS etc., leading to cellular damage and death. The signaling mechanisms underlying OS induced cell death are poorly understood. Identifying the signaling pathways not only helps to understand the mechanistic details but also identify novel therapeutic targets to manage them. The goal of the study was to unravel the signaling pathways using systems biology approach by integrating transcriptomic, preoteomic and cytomic measurements and use this knowledge towards characterization of normal and abnormal phenotypes. As a first step towards this goal, we characterized the response of human microvascular endothelial cells from lung (HMVEC-L) with hydrogen peroxide (H_2O_2). We found H_2O_2 induce cell injury through OS in a dose and time dependent manner as suggested by biochemical (LDH release) and phenotype assays (Live/Dead assays). Upon further investigations, we found that cell injury was mediated through the intrinsic pathway of apoptosis (Caspase Glo3/7 assays; Bcl2, Bax and cleaved PARP assays; mitochondrial membrane potential assays). To identify the signaling pathways invoked, we probed the canonical pathways PKA, PKB, PKC, ERK, P38 and JNK at specific time points (0, 5, 10, 15, 30 and 60 min) with and without H_2O_2 treatment by detecting their pathway specific phosphoproteins. Our study shows that H_2O_2 induce oxidative stress and cause cell injury by activating the intrinsic pathway of apoptosis. The key signaling pathways that mediate this effect are found to be in the following order of significance: P38, JNK, PKC, PKB, ERK and PKA, and they were further confirmed by Protein Kinase inhibitor studies. We are using these inputs for fine-grain analysis of the above mentioned transcriptome, proteome and cytome measurements and use systems biology approach to characterize the normal and abnormal phenotypes of OS cell signaling.

1996/B459

Dynamics of Cellular Protein B23 in Relation to Alterations in CK2 Activity in Prostate Cancer Cells.

G. Wang, K. Ahmed; Research Service/Lab Med Path, V.A. Medical Ctr/Univ of Minnesota, Minneapolis, MN

Protein B23 (numatrin), a nucleolar and nuclear matrix associated protein essential for rRNA synthesis has been shown to act as a chaperone capable of shuttling between cytoplasm and nucleus. Protein B23 is a substrate for protein kinase CK2. In previous work we showed that

nuclear B23 was completely lost in the prostate epithelial cells from androgen deprived animals associated with loss of nuclear CK2 and induction of apoptosis in the prostate epithelial cells which was presumed to be from degradation of B23 under the experimental conditions (Tawfic et al., J Biol Chem 270: 21009-21015, 1995). Here we have examined the dynamics of B23 in the rat ventral prostate and prostate cancer cells (ALVA-41 and PC-3) in which CK2 activity was altered by various manipulations. In androgen deprived rats, prostatic B23 was lost from the nuclear matrix (as observed previously); however, this was without a significant change in its cytoplasmic level. In prostate cancer cells, we observed that inhibition of CK2 by a chemical inhibitor (TBB) causes a profound reduction in the immunoreactive nuclear B23 without a significant reduction in the cytoplasmic B23. Treatment of cells with a low dose of TBB which sensitizes them to apoptosis inducing agents such as TRAIL or etoposide causes a similar loss of nuclear matrix associated B23 protein without a major effect on B23 level in the cytoplasm. Overexpression of CK2 blocked the loss of nuclear B23 following treatment with TRAIL and etoposide. We also demonstrated that B23 and CK2 were co-localized in the nucleus as examined immunofluorescence analysis of the two proteins in the cell. Thus, it appears that translocation of B23 along with CK2 from the nucleus may be an important mechanism of its reduction in the nucleus in response to modulation in CK2 and induction of apoptosis.

1997/B460

Modulation of Apoptosis Inducing Factor by Nrf2 Pathways in Neural Cells.

S. D. Crockett[1], E. Kindred[2], B. Sims[2], W. Deng[1]; [1]Human Anatomy and Cell Biology, Univ. of CA Davis, Davis, CA, [2]Pediatrics, Univ. of Alabama at Birmingham, Birmingham, AL

Objective: To determine if AIF is modulated by a known stress regulatory gene. Mammalian Apoptosis-Inducing Factor (AIF) is a 67-kDa flavoprotein with a dual function of caspase independent cell death and a lesser studied mitochondrial oxidoreductase function in normal physiological conditions. Previous studies have shown that cells with reduced levels of AIF are resistant to PARP-1-dependent cell death, PAR polymer cytotoxicity, and reduced cerebral infarct volume seen with middle cerebral artery occlusion. Other studies have linked AIF as a protein that can protect against oxidative stress via a type of antioxidant function. However, there are no known mechanistic strategies whereby the protective characteristics of this protein can be upregulated without causing indirect cellular death. The aim of our study is to show that under mildly stressful conditions a possible mechanism to enhance AIF's protective oxidoreductase properties is through the modulation of the nuclear factor E2-related factor 2 (Nrf2) pathway. Nrf2 is a known transcription factor that induces the expression of a variety of cytoprotective and detoxification genes. Methods: We used to collect preliminary results were obtained by examination of primary neural cell culture under mild hypoxic stress. Sample analysis was preformed using standard western blot, immunocytochemisty, in situ cell death detection, and GSH depletion assay. Results: AIF expression was significantly increased in neural cultures after exposure to increased concentrations of tert-butylhydroquinone, a known enhancer of Nrf2-mediated transcriptional activation. No significant increase in cell death or changes in glutathione levels were detected. Conclusion: The data suggest that AIF can be modulated by a stress related genes such as Nrf2 and an increase does not lead to concomitant cell death, but a possible protective state.

1998/B461

Effect of Resveratrol on the Tumor Necrosis Factor-a-Induced Apoptosis in Prostate Cancer Cells.

H. Lee, H. Byun, M. Won, K. Park, Y. Kim, B. Choi, Y. Li, J. Hong, J. Seok, G. Hur; Pharmacology, Chungnam National University, Daejeon, South Korea

Resveratrol, a polyphenolic phytoalexin, possesses chemopreventive properties against several cancers, the molecular mechanism by which it inhibits cell growth and induces apoptosis. Recent studies have provided support for the use of resveratrol in human cancer, with combination with either chemotherapeutic drugs or cytotoxic factor. Here, we examined the molecular mechanisms of resveratrol and its interactive effects with tumor necrosis factor-a (TNF-a) on apoptosis in androgen-sensitive (LNCaP) and androgen-insensitive (PC3) prostate cancer cells. However, these tumor cells were found to be TNF-a resistant, the combination of resveratrol and TNF-a enhanced the apoptosis. Resveratrol treatment resulted in significant activation of AMP-activated protein kinase (AMPK), p53 and induced the cell cycle arrest, which may occur at various stages of apoptotic pathway. Resveratrol cotreatment with TNF-a enhances activities of caspase-8, caspase-3 and down-regulates the antiapoptotic protein Bcl-2, XIAP and results in the decrease of mitochondrial membrane potential and potentiates activation of c-Jun NH2-terminal kinase (JNK) and inhibits nuclear factor-kB activity. Our data suggested that the resveratrol sensitization effect in TNF-a resistant pancreatic cells was mediated through AMPK pathway, requires p53 activity and involves mitochondria-dependent apoptotic cascades. This work was supported by grant from the National R&D Program for Cancer Control Ministry of Health & Welfare, Republic of Korea (NO: 0720560), and R13-2007-020-01002-0 of the Korea Science and Engineering Foundation through the Infection Signaling Network Research Center at Chungnam National University. Key words: Resveratrol, TNF, Apoptosis, AMPK, Mitochondrial damage

1999/B462

Expression of the Tenascin-C by Asian Sand Dust (ASD)-Particulate Matters (PM) on C57BL/6 Mice Liver In Vivo.

M. Seo, Y. Kwon, S. Choi, Y. Lee, M. Bae, S. Choi, E. Lee, D. Kim, Y. Hwang; Division of Biological Science, Gachon Univ. of Medicine & Science, Incheon, South Korea

Wind erosion in arid and semi-arid areas of middle and northwestern China forms the Asian sand dust (ASD)-PM aerosol. This ASD-PM spreads over large areas, including East China, Korean Peninsula, and Japan. Exposure to fine particle from ASD-PM has caused a variety of health problems, not limited to sore throat and asthma in otherwise healthy people and even if oxidative DNA damage in the respect of molecular biology. However, the data concerning the fibrotic effects of ASD-PM event particles remain unclear. Transforming growth factor-beta (TGF-β) isoforms are multifunctional cytokines that play a central role in wound healing and in tissue repair process. Also, this cytokine regulates the expression of many matrix proteins including fibronectin, tenascin, and decorin etc. Among these proteins, Tn-C is a multidomain large extracellular matrix glycoprotein and is up-regulated during liver fibrotic processes. According to the functions of TGF-β and Tn-C, we studied the expression of them as an aspect of interaction affecting on the liver fibrosis. Exposure to ASD-PM to C57BL/6 mice did three times a week for 4, 8, 12 weeks, each times, 20ul of 0.1, 0.2, 0.4 mg/mL autoclaved ASD-PM suspension and saline (negative

control) via intra trachea instillation. Mice were sacrificed after they were injected 12, 24, 36 times by ASD-PM. One part of the liver was treated for checking the expression of Tn-C by western blotting and the other part was detected for transcription mRNA by RT-PCR, targeted TGF-β and Tn-C. The result of western blotting, the expression of Tn-C showed us ambiguous change on samples. However, we demonstrate the changes of expression level of TGF-β and Tn-C by RT-PCR. Moreover, we find out the correlation between TGF-β and Tn-C expression on the liver fibrosis. These results suggest ASD-PM interrupted transcription of Tn-C onset that related to the liver fibrosis.

Imaging Technology II (2000 – 2016)

2000/B464

Tetra-Calcium Phosphate Is Used in Crustacean Cuticle Mineral Chemistry.

J. G. Kunkel[1], M. J. Jercinovic[2], W. Nagel[3]; [1]Biology, UMass Amherst, Amherst, MA, [2]Geosciences, UMass Amherst, Amherst, MA, [3]Physiology, Ludwig Maximilian University, Munich, Germany

The dermal gland canals and neuron channels through lobster cuticle are lined with a phosphatidic compound that has the calcium phosphate ratio of tetra-calcium phosphate. This is the first report of a general use for the precursor to apatite in a crustacean. The calcium, magnesium, barium, phosphorous, chloride, fluoride, iron, and copper content of American lobster, Homarus americanus, freeze substituted cuticle was determined at a sub-micron resolution using X-ray backscatter analysis. Standards of calcite, apatite and crystalline monobasic calcium phosphate were used to establish the well known ratios of calcium and phosphorous in our resin embedding protocol. The raster images of 0.24 micron diamond polished gland canals and neuron channels were produced with a Cameca Ultrachron Electron Microprobe. The raster images of calcium and phosphate content clearly show that a dense layer of calcite covers the outer layer of the cuticle interface facing sea water but this exocuticle surface layer is replaced with a thin layer of tetra-calcium phosphate lining the gland and neuronal canals through the cuticle. This layer of a calcium phosphatidic compound provides a less soluble barrier to bacterial attack in vulnerable small spaces. In addition, tetra-calcium phosphate is known to convert to apatite in a thin surface layer releasing antibiotic hydroxyls which would add to a protective antibiotic function. Supported by grants from MIT SeaGrant and the Rhode Island SeaGrant Lobster Health Initiative.

2001/B465

Gauging Cytotoxicity via Nuclear Morphometry in Chemical Library Screening.

B. Azimi[2,1], I. Kalcheva[1], S. Heynen-Genel[1], J. H. Price[1]; [1]The Burnham Institute for Medical Research, La Jolla, CA, [2]University of California San Diego, La Jolla, CA

Detection and quantification of compound toxicity has become an important challenge in identifying drug candidates in small molecule screening. The early detection of toxic compounds in the drug development pipeline can significantly reduce cost and development time by helping to identify compounds that may have cardiac, hepatic and renal toxicity as drugs. Ideally, analysis of toxicity should be multiplexed with drug screening to simultaneously acquire toxicity and assay target data. Other than evaluating DNA content, however, toxicity measurements rely on antibodies specific for markers involved in cell death. With the need for antibodies, and their inherent high cost, multiplexing such toxicity assays is unfeasible for large chemical libraries (i.e. multiple hundreds of thousands of compounds). With the advent of image-based high-content screening, a wealth of whole-cell, nuclear and organelle morphometric and fluorometric measurements, are available for analysis and data mining. The images acquired can help resolve details of the cell that indicate the early events in cell death, including nuclear fragmentation and DNA condensation. Therefore, we hypothesize that these features can be used to extract toxicity data and mimic data obtained from antibody staining of cell death markers. Here, an a priori set of nuclear morphology features, including nuclear area, shape and chromatin condensation in the nucleus are combined with cell count to calculate a single cytotoxicity index. This cytotoxicity index is verified using dose response and IC50 of known toxic compounds (e.g. Taxol) on J774 and Hela cells. Moreover, a retrospective study of high content screening data was performed by mining toxicity data and verification across multiple molecular library screening campaigns.

2002/B466

Single-Molecule Motions of Oligoarginine Transporter Conjugates on the Plasma Membrane of CHO Cells.

H. Lee[1], E. Dubikovskaya[1], R. Twieg[2], P. Wender[1], W. Moerner[1]; [1]Chemistry Department, Stanford University, Stanford, CA, [2]Department of Chemistry, Kent State University, Kent, OH

Many strategies have been developed to cross plasma membranes and deliver exogenous molecules. Guanidinium-rich cell-penetrating peptides(CPPs) have emerged as promising molecular transporting vectors. While many mechanisms have been proposed to explain its cell entry, due to various obfuscating factors, the current picture of CPP cellular entry remains complex. To examine the behavior of guanidinium-rich CPPs on a biological membrane, single fluorescently-labeled oligoarginines were imaged on the plasma membrane of Chinese hamster ovary cells. The diffusional motion on the membrane, characterized by diffusion coefficient and residence time, defined as the time from the initial appearance of a single-molecule spot on the membrane to the time the molecule disappears from the imaging focal plane, was observed for a fluorophore-labeled octaarginine and compared with the corresponding values observed for tetraarginine conjugates, lipid analogs, and Transferrin(enter cells through endocytosis). Imaging of oligoarginines was enabled by a novel fluorophore in the dicyanomethylenedihydrofuran (DCDHF) family, which brightens upon interaction with the plasma membrane at normal oxygen concentrations. Single-molecule imaging at the plasma membrane shows that the lateral motions of the octaarginine conjugates on the plasma membrane are highly heterogeneous, and cannot be described as Brownian motion with a single diffusion coefficient, the behavior observed for the lipid analog. Furthermore, while the octaarginine conjugate behavior shares some common features with endocytosis, the two also exhibit dissimilar traits when diffusional motions and residence times of single constructs are compared. Also, pretreatment of cells with cytochalasin D, an actin filament disruptor, produces no significant effect, which further rules out unimodal endocytosis. Also, the involvement of membrane potential in octaarginine-membrane interaction is supported by significant changes in the motion with high [K+]

treatment. This study indicates that the mode by which the octaarginine penetrates the cell membrane appears to either be a multi-mechanism uptake process or a mechanism different from unimodal passive diffusion or endocytosis.

2003/B467

Automated Analyses of Migration and Differentiation in Cultured Human Embryonic Stem Cells.

D. Calzolari[1,2], M. Ranji[1], N. Prigozhina[1], K. Wei[1], M. Mercola[1], J. H. Price[1]; [1]Burnham Institute for Medical Research, La Jolla, CA, [2]Universita' di Modena e Reggio Emilia, Modena, Italy

Abstract during normal embryonic development, hESC follow a remarkably coordinated migration and differentiation program, many details of which remain a mystery. Additionally, hESC are actively researched for clinical applications. The ability to effectively visualize large numbers of fluorescently labeled, functional differentiating hESCs during their migration as they form complex 3D structures is important for a better understanding of the interplay between cellular migration and differentiation. Here we use fully automated techniques to study the formation of these biological units, and show "migration maps" created from time-lapse movies of hESCs expressing H2B fused with fluorescent proteins. Methods Expression of H2B-fused, nuclear-localized eGFP and mCherry proteins allow visualization of DNA content as well as cell tracking. We developed automated tools to track the migration of specific lineages of interest during hESC differentiation. Transgenic hESC lines have been created that employ cardiac-specific (αMHC) and endothelial-specific (Tie-2) promoters driving eGFP and mCherry, respectively. Cell type-specific expression of fluorescent reporter activity allows us to track these cell types throughout the differentiation process. Results We have preliminary data demonstrating that both cardiomyocytes and endothelial cells arise during hESC differentiation. We present a quantitative analysis on different physical properties (such as velocity, acceleration, population distribution, distance traveled, cosine and sine) of the cell migration both referred to the single cell and to the group behavior (correlation measurement). Conclusion simultaneously tracking cardiomyocytes and endothelial cells would provide a method of understanding how these cell types physically associate in vitro. This is of particular interest due to the intimate relationship of these cell types in embryonic development as well as in adult myocardium and would shed light on the organizational influences of these cell types during development.

2004/B468

Multiple Labeling of Membrane Proteins with Newly Developed Nanoparticles.

A. R. Loukanov, N. Kamasawa, R. Danev, R. Shigemoto, K. Nagayama; Department of Physiological Science, National Institutes of Natural Sciences, Okazaki, Japan

We are developing an electron microscopic technique for localization and quantification of different species immunolabeled membrane proteins which remain scattered on freeze-fractured replica film. To achieve higher labeling efficient and spatial resolution three kinds of glutamate receptors (NMDA, AMPA and d2 in hippocampal and cerebellum area) were labeled with 3-5 nm nanoparticles (NPs) contain different elements (respectively Au, Cd and Zn). To recognize such small inorganic labels, the replica film was made only with carbon to avoid the background usually seen in the platinum-carbon replica. Then 200 kV scanning transmission electron microscope equipped with an energy disperse X-ray (EDX) spectrometer was employed to observe the carbon-replica's ultrastructural feature and to localize NPs in individual synapses. The NPs were distinguished from each other by EDX microanalysis of their chemical composition. The collected data enables to measure the receptor distribution in the active zones.

2005/B469

Development of Imaging System Using RNA Multiaptamers for Real-Time Gene Expression.

I. Shin, M. Ilgu, E. Haarberg, M. Nilsen-Hamilton; Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA

Reporter proteins, such as GFP are the major reporter system to detect real-time gene expression. After cloning and expression of GFP, non destructive real time in vivo imaging is possible. However, GFP as a protein, has higher energy cost for synthesis and exhibits a long delay between stimulus and expression than RNA. To establish an imaging system with a lower energy cost to the cell and a faster response time than available with protein reporter systems, we are developing an RNA imaging system, called IMAGEtag (Intracellular MultiAptamer Genetic tag), which utilizes strings of aptamers that are expressed from a promoter of choice. To develop this reporter system, the available RNA aptamers for tobramycin, neomycin and theophylline are being used and their targets have been labeled with either fluorescent molecule or radioisotope for imaging. Vectors that express multiple repeated aptamer sequences have been developed to increase the aptamer concentration in the cell and thus also increase signal/noise for imaging. To further increase signal/noise, aptamer targets are separately labeled with fluorescent FRET pairs to decrease the background noise. The reporter system is currently being developed for reporting gene expression changes in yeast cells. Once optimized for imaging in cell culture, the IMAGEtag reporters will have many possible imaging applications, including real-time detection of changes in gene expression and mRNA processing, and tracking RNA movements in cells.

2006/B470

Stable and Non-Disruptive In Vitro/In Vivo Labeling of Mesenchymal Stem Cells by Internalizing Quantum Dots.

Y. Ohyabu[1,2], Z. Kaul[1,3], T. Yoshioka[1,4], K. Inoue[1,4], S. Sakai[4], H. Mishima[4,1], T. Uemura[1], S. C. Kaul[1], R. Wadhwa[1]; [1]Nanotechnology Research Institute, AIST(Natl Inst. Adv. Industr. Sci. Technol.), "Tsukuba, Ibaraki", Japan, [2]Tokyo Institute of Technology, Tokyo, Japan, [3]Childrens Medical Research Institute, Westmead, NSW, Australia, [4]University of Tsukuba, Tsukuba, Japan

Mesenchymal stem cells (MSCs) can differentiate into a variety of mesodermal lineage cell types (myocytes, adipocytes, chondrocytes and osteocytes) and tissues. For both basic and clinical research, it is necessary to study these cells under in vivo environment, which can be facilitated by cell labeling technologies that can distinguish implanted cells from the host tissues. Recently, quantum dots (QDs) have emerged as high-quality fluorescent alternatives to conventional dyes. We initially generated internalizing quantum dots (i-QDs) by conjugating them to a unique internalizing antibody against a hsp70 family stress chaperone, mortalin, that is upregulated and expressed on the surface of dividing cells. MSCs from rat, rabbit, monkey and human were all labeled efficiently with i-QDs with almost 100% efficiency. We found that the rat i-MSCs underwent osteogenesis and adipogenesis with the same efficiency and time course as the c-MSCs(control). There were no differences in the Alizarin Red staining intensities at one or two weeks of differentiation. Similarly, Oil Red staining revealed

that the i-MSCs were not affected in their adipogenic potential. We next used cultured bone transplantation models to examine the effect of i-QDs on the differentiation potential of MSCs in vivo. In both rat and rabbit models, we found that the c-MSCs and i-MSCs differentiated to form bone matrix with the same efficiency and time course. The results suggested that the regenerated bone in the porous area of the scaffold was mostly derived from implanted donor cells and their derivative populations. Furthermore, thick endochondral and osseous tissues were observed in the deeper portion of the reparative region demonstrating clear bone formation in vivo. Importantly, the chondrogenic differentiation of c-MSCs and i-MSCs was equally efficient and followed the same time course. From these data, it was concluded that the i-QDs can serve as a non-toxic, genetically noninvasive, and functionally inert, sensitive long-term imaging tool for in vitro and in vivo studies in multiple mammalian species.

2007/B471

PSICIC: a Quantitative Image Analysis Toolkit That Reveals Noise and Asymmetry in Bacterial Division.

J. Guberman, N. Wingreen, Z. Gitai; Molecular Biology, Princeton University, Princeton, NJ

Quantitative image analysis is an important part of cellular imaging, providing both enriched information about individual cell properties and the ability to analyze large datasets. We have developed a generally-applicable, standardized, and modular software suite termed Projected System of Internal Coordinates from Interpolated Contours (PSICIC) that solves two common problems in image quantitation. Specifically, we use interpolated contours to define cell borders with nearly an order of magnitude greater accuracy and precision than traditional approaches, and we establish an internal coordinate system for each cell that can be readily superimposed to facilitate comparisons among cells. Though PSICIC can be readily applied to any cell type, it is particularly well suited to analyzing small cells such as bacteria. We have thus initially focused on two outstanding problems in bacterial cell biology. We have used PSICIC to establish that the cell-fate determinant, SpoIIE, is asymmetrically localized during *Bacillus subtilis* sporulation, thereby demonstrating the ability of PSICIC to discern protein localization features at sub-pixel scales. By exploiting the ease of large-scale automated analysis with PSICIC, we also examined the accuracy of the symmetric division of *Escherichia coli* with unprecedented data density. Our results demonstrate that *E. coli* populations divide with extreme accuracy and identify a new role for the Min system in regulating division-site placement throughout the cell length, but only prior to the initiation of cell constriction. In addition to contributing to the understanding of sporulation and division, our analyses of SpoIIE asymmetric localization and *E. coli* division-site accuracy are intended to highlight multiple aspects of the power of PSICIC in analyzing data from different species, extracting single-cell and population statistics, and utilizing both morphological and protein localization information.

2008/B472

Single-Cell Molecular Profiling Reveals the Origin of Cell-To-Cell Phenotypic Variability in Adipogenesis.

T. T. Le[1], J. Cheng[1,2]; [1]Weldon School of Biomedical Engineering, Purdue University, West Lafayette, IN, [2]Department of Chemistry, Purdue University, West Lafayette, IN

Cell-to-cell phenotypic variability within an isogenic cell population poses significant challenge to drug treatment. Cells with distinct molecular profiles exhibit distinct phenotypic behaviors. Hence, drug treatments targeting a heterogeneous cell population often have varied intended effects. To improve treatment efficacy, the sources of cell-to-cell variability should be identified and suppressed. However, unlike genetic mutation which can be identified with sequencing, non-genetic cause of cell-to-cell variability cannot be readily investigated. Standard population measurement techniques describe average behavior and are insufficient to investigate variability among cells. To describe the molecular cause of phenotypic variability, cellular content and phenotypic behavior must be measured simultaneously at the level of single cells. Here, we describe a single-cell molecular profiling approach to investigate the origin of cell-to-cell phenotypic variability during adipogenesis in 3T3-L1 cells. Using the multimodal capability of a nonlinear optical (NLO) microscope, we simultaneously image insulin signal (input), expression level of adipogenic marker genes, pparγ and pref-1 (decision), and lipid droplets (output) within single 3T3-L1 cells. Flow cytometry analyses further quantitatively characterize the input-decision-output process in single cells. We discover a strong correlation between insulin utilization capability and the size of lipid droplets and identify the kinetics of insulin signal processing as the origin of variability in adipogenic activity among 3T3-L1 cells. We find that multimodal NLO imaging, together with flow cytometry, present a powerful means to study cell-to-cell phenotypic variability. It is conceivable this single-cell molecular profiling approach can be applied to other cellular processes to uncover properties inaccessible to standard population measurements. Furthermore, identification of the source of phenotypic variability among cells should present new opportunity for better drug treatment design.

2009/B473

Real-Time Movement of Nutrients in a Living Plant.

T. M. Nakanishi; Graduate School of Agricultural and Life Sciences, University of Tokyo, Tokyo, Japan

To study living plant activity, nondestructive imaging method is very important. Recently, imaging technique was drastically developed and most of them are based on fluorescent dyes or probes. However, imaging with fluorescent chemicals has disadvantage that it is very difficult to determine the amount of ions or chemicals fluorescence indicates. To solve this problem and also aiming nondestructive imaging, we developed an imaging system, measuring beta-ray from the isotope, since beta-ray counting indicates the direct amount of ions or chemicals. Among the application of isotopes, imaging with positron emitters is known as PET (Positron Emission Tomography), which was well developed in medical field. However, PET measures a pair of gamma-rays emitted from the sample, therefore, the resolution cannot be higher than several mm, in order. In the case of beta-ray imaging, resolution can be much higher than that of gamma-ray imaging. The system we developed was consisted of MCP (Multi-Channel Plate) deposited with scintilator, which converts beta-ray to light, and the light is guided to a highly sensitive CCD camera. Using the systems, any beta-ray emitters commercially available, such as 14C, 45Ca, 32P, etc., except for 3H, were visualized when applied to a plant sample. After studying various kind of scintolator and the depth of deposition, the sensitivity of beta-ray emitter, for example 14C, was about 10 times higher than that obtained by an imaging plate (IP). When 32P was applied to culture solution of a soybean plant, 32P ion movements in culture solution as well as that in a plant were visualized successively. There was a preferential movement of 32P in up-ground part of the plant, which tendency was different among the tissues. In the case of 14C-labeled

amino acid, glutamine, movement and concentration of this amino-acid towards root in culture solution was shown. It was observed that there was a rhythmic movement in uptake manner of amino-acid by root. Through image analysis, the movement of amino-acid within any part of the root was analyzed. The resolution of the image is now about 0.1mm.

2010/B474

Development of New Fluorescent Proteins: a Bright Blue mTagBFP and the Changing Color from Blue to Red Fluorescent Timers.

O. Subach[1], F. Subach[1], K. Morozova[1], K. Piatkevich[1], A. Cuervo[2], V. Verkhusha[1]; [1]Department of Anatomy and Structural Biology, and Biophotonics Center, Albert Einstein College of Medicine, Bronx, NY, [2]Department of Developmental and Molecular Biology, and Institute for Aging Research, Albert Einstein College of Medicine, Bronx, NY

Kinetic pathway for the red chromophore formation, in which an anionic red chromophore is formed from the neutral blue chromophore form, provides a basis to develop two types of fluorescent proteins (FPs) from available red FPs. The first type is bright blue fluorescent proteins (BFPs) with a tyrosine residue in the chromophore. The second type is fluorescent timers (FTs), which change their fluorescence spectrum from blue to red over time. To develop BFPs, a rational design strategy was applied to five red FPs of the different genetic background such as TagRFP, mCherry, HcRed1, M355NA and mKeima, which all were converted into blue probes. Further improvement of the TagRFP's blue variant using random mutagenesis resulted in an enhanced monomeric protein, called mTagBFP, with the excitation/emission maxima at 399/456 nm. The mTagBFP is characterized by the 1.8-fold higher brightness, the faster chromophore maturation and the better pH stability than currently available BFPs with a histidine residue in the chromophore. To develop FTs, the rational design followed by random mutagenesis was applied to mCherry. The resulting monomeric FTs exhibit the distinctive fast, medium and slow blue-to-red chromophore maturation rates that depend on the temperature. At 37^0C the maxima of the blue fluorescence were observed at 0.25, 1.2 and 9.8 hours for the Fast-FT, Medium-FT and Slow-FT, respectively. The half-maxima of the red fluorescence were reached at 7.1, 3.9 and 28 hours, respectively. The Medium-FT allowed for tracking of an intracellular trafficking of the newly synthesized lysosome-associated membrane protein type 2A (LAMP-2A) and determination of an age of the FT-fused protein in the targeted compartments. Our data indicates the LAMP-2A transport through the plasma membrane and early/recycling endosomes to lysosomes is a major pathway for the LAMP-2A trafficking. The spectral properties of mTagBFP and FTs allow for their simultaneous use with green FPs in multicolor cell labeling and in FRET applications. Lastly, the blue-to-red FTs provide a reliable way to analyze spatial-temporal history of the FT-fused proteins, to determine their age, and to study activation and repression of their synthesis and degradation.

2011/B475

High Resolution Imaging of Live Single Cell Surface Change upon Fixation Using AFM and SICM.

N. Cho[1], E. Kim[2], G. Jung[2], S. Cho[2]; [1]School of Medicine, Stanford University, Stanford, CA, [2]Advanced Research, Park Systems, Suwon, South Korea

Investigating a surface morphology of single cell membrane in vivo with optical techniques is extremely difficult due to the transparency and resolution limitation. Atomic Force Microscopy (AFM) can give nm resolution and is not affected by the transparency of the cell since images are recorded from the position of the probe which is gliding over the membrane. However, AFM may not be very convenient tool for imaging soft cell surface in vivo because of interactions with extracellular matrix including glycoprotein and carbohydrate. A new ion conductance microscope (ICM) module developed from a conventional Atomic Force Microscope (AFM) platform, has been able to image live cell membrane in high resolution by using a glass micropipette as a sensitive probe instead of silicon based stylus. A glass micropipette as an ICM probe filled with electrolyte senses ion current to feedback its position when it images samples immersed in buffer solutions. The ICM images the sample in liquid without physical contact. Live Cos-1 Cell and Stem Cell membrane surface were observed in high resolution then changes upon fixation were examined continuatively using ICM and AFM. We have observed the disappearance of cilia like structures on cell surface after fixation. High disturbance in AFM imaging due to the tip-sample interaction was also greatly diminished after fixation. Elasticity comparison between live and fixed cells was made through the AFM force-distance mapping technique. The presenting technology here can be utilized to extracellular domain of any cell type. AFM and ICM together with optical microscopic technology offer immense possibilities to investigate dynamic and biophysical properties of live cells in nanometer scale.

2012/B476

Single-Molecule Fluorescence Imaging with Sub-Nanometer Resolution.

A. Pertsinidis[1,2], Y. Zhang[2], S. Chu[3]; [1]QB3 Institute, UC Berkeley, Berkeley, CA, [2]Physics, Stanford University, Stanford, CA, [3]Lawrence Berkeley National Laboratory, Berkeley, CA

Objective: The emerging field of fluorescence nano-scopy promises to revolutionize biomedical research, and remarkable progress has been made in the measurement of nano-meter distances. Although diffraction of visible light limits the far-field optical resolution to ~200nm, the center position of spatially resolved fluorescent molecules or nano-particles can be located to much higher precision. Notably, in high resolution microwave and optical spectroscopy there are numerous examples where the line-center is determined with a precision of less than 0.000001 of the line-width. In contrast, the brightest single fluorescent emitters can be detected with a Signal-to-Noise-Ratio of ~100, limiting the localization precision to 0.01 (~1.5nm) of the microscope Point-Spread-Function (PSF) width. Moreover the error in co-localizing two or more single emitters is notably worse, remaining greater than 0.03-0.05 (5-10nm) of the PSF width. Results: Using an actively stabilized imaging setup and feedback control on the position of individual fluorescent molecules we achieve two-color single-molecule imaging with 0.5nm absolute localization and registration accuracy. To suppress Brownian motion in solution we employ optical trapping to lock the position and orientation of a surface tethered DNA molecule. This allows us to resolve with 0.7nm absolute accuracy distances between two different color dye-molecules attached at known positions along the DNA molecule. The statistical uncertainty in the mean for an ensemble of N~10 identical single molecule samples is limited only by the total number of collected photons to ~0.3nm, or ~0.002 of the width of the optical PSF. Conclusion: We demonstrate sub-nanometer resolution in measurements of molecular-scale distances using conventional far-field fluorescence imaging optics, at room temperature and in physiological buffer conditions. Our method can be used to improve the resolution of many sub-wavelength, far-field imaging methods such as those based on co-localization of stochastically

excited molecules. The improved resolution will allow deciphering in real-time, at the single molecule level the structure and dynamics of large, multi-subunit biological complexes.

2013/B477

Multilayer Three-Dimensional Super-Resolution Imaging of Thick Samples.

A. Vaziri, J. Tang, H. Shroff, C. Shank; Howard Hughes Medical Insititute, Ashburn, VA

Recent advances in optical microscopy have created the capability of creating images in biological samples beyond the diffraction limit at nanometer resolution. A general feature of most of the techniques based on photoactivated localization microscopy (PALM) or stochastic optical reconstruction microscopy (STORM) has been that the imaging depth is limited to a fraction of an optical wavelength. However, in order to study whole cells or organelles deep into the cell, the extension of these methods to a 3D super-resolution technique is required. We report an advance in optical microscopy that enables imaging of protein distributions in cells with a lateral localization precision better than 50 nm at multiple imaging planes deep in biological samples. The approach is based on combining the lateral super-resolution provided by PALM with two-photon temporal focusing that provides optical sectioning. We have generated super-resolution images over an axial range of ~10μm in both mitochondrially-labeled fixed cells, and in the membranes of living S2 Drosophila cells. Given the fact that temporal focusing makes optical sectioning over a range of many tens of microns possible, this technique can be expected to become a method of choice when single-molecule resolution in larger volumes is required. Areas which could benefit from temporal focusing-PALM include developmental biology on molecular level or in neuroscience studies using drosophila where questions of neural circuitry could be addressed on the level of whole brains.

2014/B478

Seeing the Unseen in a Cell with Super-Resolution Fluorescence Microscopy.

B. Huang[1,2], M. Bates[3], W. Wang[4], S. A. Jones[2], B. Brandenburg[1,2], G. T. Dempsey[5], X. Zhuang[1,2,4]; [1]Howard Hughes Medical Institute, Harvard University, Cambridge, MA, [2]Department of Chemistry and Chemical Biology, Harvard University, Cambridge, MA, [3]School of Engineering and Applied Sciences, Harvard University, Cambridge, MA, [4]Department of Physics, Harvard University, Cambridge, MA, [5]Graduate Program in Biophysics, Harvard University, Cambridge, MA

As one of the most powerful imaging techniques for studying cellular processes, fluorescence microscopy allows noninvasive imaging of live samples with molecular specificity. However, the spatial resolution of conventional fluorescence microscopy is limited by the diffraction of light to about 200-300 nm in the lateral directions and 500-700 nm in the axial direction, leaving many biological structures too small to be observed in detail. To overcome this limit, we have developed the Stochastic Optical Reconstruction Microscopy (STROM) technique. By determining the position of individual photoswitchable probe molecules, it has achieved a 20-30 nm lateral resolution in cellular samples. The incorporation of three-dimensional (3D) single molecule localization further enables 3D STORM of a whole cell with 50-60 nm axial resolution. We have also created photoswitchable fluorophores for multicolor imaging by combinatorial pairing of various activator dyes and reporter dyes. We have demonstrated the ability of STORM to visualize spatial relationships unresolvable by conventional fluorescence microscopy, including organelle-cytoskeleton interactions in cells and synaptic structure organizations of the olfactory system in brain sections.

2015/B479

The Use of High Content Analysis Techniques to Quantify the Effects of Sirna's on Lipid Droplets and Lipid Droplet-Associated Proteins in Hepatocyte-derived Cell Lines.

P. A. Loy[1], E. Suyama[2], P. Aza-Blanc[2], P. McDonough[1]; [1]Vala Sciences Inc, San Diego, CA, [2]Burnham Institute for Medical Research, La Jolla, CA

Lipid droplets (LDs) form in a variety of cell and tissue types, including adipocytes, skeletal muscle cells, and hepatocytes, and the mechanisms regulating LD formation are incompletely understood. Fatty liver disease is a condition in which hepatocytes contain LD in excessive amounts, and is a precursor condition for fibrosis, cirrhosis, and liver cancer and fatty liver disease is becoming more prevalent population becomes more obese. To further our understanding of LD formation, and to enable screens to identify chemicals or genomic material (cDNA, siRNA, or microRNAs) that may influence LDs in hepatocytes, Vala Sciences Inc is developing high content analysis (HCA) methods, based upon cultured cells, robotic digital microscopy workstations, and cell image analysis software, to analyze of LDs and associated proteins in hepatocyte-derived cell lines. Huh-7 cells (derived from human hepatocytes) were plated in a 384-well/plate format, and transfected with 3 siRNA species (si RNA #1, #2, and #3) directed towards ADFP (aka adipophilin, ADRP) a LD-associated protein related to perilipin. Following a 3 day culture period, the cells were fixed and visualized for nuclei, LDs, and ADFP, the plates scanned in 3 fluorescence channels (blue, nuclei; green, lipid droplets; red ADFP) utilizing a 20X objective to collect images from 4 fields of view per well. Images were then quantified, on a cell-by-cell basis for LDs and ADFP expression utilizing Vala's CyteSeerTM cell image analysis program. Non-transfected cells, cells exposed to only the transfection reagent, and cells exposed to the transfection reagent plus non-specific siRNA expressed very similar amounts of ADFP and LDs. In contrast, siRNA#1, #2, and #3 reduced ADFP expression by 69%, 63%, and 13%, and reduced lipid droplets by 55%, 9%, and 19%, respectively. The results are consistent with the hypothesis that ADFP is involved in hepatic LD formation, and illustrate that HCA techniques can be applied to this critical problem for human health.

2016/B480

Analysis of Ras Interactome in Saccharomyces Cerevisiae Using Bimolecular Fluorescence Complementation Assay.

E. Yang, W. Huh; School of Biological Sciences, Seoul National University, Seoul, South Korea

It is well known that activation of diverse cell-surface receptors can stimulate convergent signals that lead to activation of Ras. Once activated, Ras is believed to activate a simple linear cascade of cytoplasmic kinases to complete the link between the cell surface and the nucleus. However, recent studies have established that much more Ras effectors than believed are present in cells and activated Ras stimulates a multitude of downstream signaling cascades. To identify novel Ras effectors based on protein-protein interaction, we constructed

a yeast VN fusion library that consists of 922 strains expressing C-terminally VN-tagged membrane proteins. 922 VN-tagged strains were then mated with a strain expressing N-terminally VC-tagged Ras2, the major Ras protein in Saccharomyces cerevisiae. Through analysis of all the resulting diploid cells with bimolecular fluorescence complementation (BiFC) assay, we found 77 proteins showing positive BiFC signals. We hope identification of these 77 putative Ras interactors will provide deeper understanding of Ras signaling pathways and other cellular functions such as proliferation, cell growth, gene expression and protein synthesis.

New and Emerging Technologies for Cell Biology II (2017 – 2034A)

2017/B481

Protein Micro-Nanoarrays on Solid and Soft Supports.

D. Aydin[1,2], M. Schwieder[1,2], I. Louban[1,2], J. P. Spatz[1,2]; [1]Department of New Materials and Biosystems, Max-Planck-Institute for Metals Research, Stuttgart, Germany, [2]Department of Biophysical Chemistry, University of Heidelberg, Heidelberg, Germany

Lateral clustering of cellular transmembrane receptors is a common mechanism to regulate signaling activity upon stimulation with the respective ligand. Recently, novel synthetic interface have been developed to control the lateral position of single proteins and mimic this situation *in vitro*. We used block-copolymer micelle nanolithography to arrange nanometer-sized gold particles in a hexagonal pattern on glass or silicon supports. These particles serve as anchor points for site-selective immobilization and controlled separation of single proteins. To distinguish effects originating from the local spacing of the ligands from those caused by their overall density, micrometer-sized patches of nanopatterns surrounded by non-structured areas can serve as a versatile control. Therefore, we combined micellar nanolithography and conventional photo or electron-beam lithography to fabricate square-centimeter-sized micro-nanopatterns. When using these substrates as templates, we are able to control single protein positioning on two different length scales. Interprotein spacing is tunable between 20 and 200 nm, while geometric arrangement of the nanoarrays is only limited by the constraints of conventional resist lithography. However, many cellular processes are not exclusively governed by biochemical signals but also by mechanical stimuli, which are strongly depending on the mechanical features of the microenvironment. The process of micro-nanostructuring of proteins was therefore extended to polymeric substrates with adjustable mechanical properties. Particularly, the elastic moduli of these synthetic materials can be tuned to mimic the extracellular space in all kind of tissues. In conclusion, we present a biomimetic interface with independent control over protein-protein distance at the nanometer scale, the geometric arrangement of protein nanoarrays and the mechanical properties of the material.

2018/B482

Patterning of Living Cells Utilizing Electrostatic Inkjet Phenomena.

S. Umezu, T. Kitajima, H. Murase, K. Katahira, H. Ohmori, Y. Ito; Advanced Science Institute, RIKEN, Wako-shi, Japan

The object of this study is to fabricate living cell structures utilizing electrostatic inkjet phenomena. It is preferable to perform laboratory experiments with precise cell structures in tissue engineering and artificial organ. We have investigated mechanism and fundamental characteristics of the electrostatic inkjet phenomena. The phenomena have two merits, higher resolution than commercial printer and ability to eject with highly viscous liquid. An experimental set-up was constructed to print cell structures utilizing electrostatic inkjet phenomena. A capillary tube made of silica coated by polyimide (I.D.: 100 micron meters, O.D.: 170 micron meters) was equipped with a bottom of a syringe. The tube filled with the liquid which contained cells was hanged down perpendicular to a dish filled with medium and growth factor. Voltage was applied between the syringes and the dish by power supplies (voltage range: -5kV ~ +5kV, Matsusada Precision Inc, Tokyo, HVR-10P). The air gap was adjusted by a z-stage and the dish was moved in x and y directions with two linear motors. Voltage application and motion of linear stages were controlled by PC. The width of cell line was less than 100 micron meters. Cells were living in spite of high voltage application because current was not flowed through cells but around cells.

2019/B483

Measurement of Mitochondrial Membrane Potential in Lymphocytes from Patients with Chronic Kidney Disease: Application of the Live Cell Array™.

V. Levy[1,2], M. Rao[3], C. Demello[3], V. Balakrishnan[3], O. Shirihai[2]; [1]Molecular Cytomics Inc., Boston, MA, [2]Molecular Medicine, Boston University, Boston, MA, [3]Nephrology, Tufts Medical Center, Boston, MA

The uremic syndrome is characterized by abnormalities in energy metabolism; insulin resistance, dyslipidemia and protein energy malnutrition. Previous data suggest a role for mitochondrial dysfunction in uremic syndrome and that mitochondrial function is compromised in multiple tissues. Here we aim at identifying a surrogate marker for mitochondrial dysfunction in blood samples. We hypothesize that in uremic patients the mitochondria in blood cells have abnormal respiratory function that can be assessed by measuring changes in Mitochondrial Membrane Potential (MMP). The Live Cell Array (Molecular Cytomics Inc.) enables the performance of functional studies of living cells at the resolution of a single cell, each cell in the array having a designated address. The array structure confines each cell to a picowell thus allowing repeated imaging of the same cells. The Live Cell Array slides were used to measure MMP in primary Lymphocytes and compare mitochondria function between patients with chronic kidney disease (CKD) and end stage renal disease (ESRD), and healthy volunteers (HV). Lymphocytes were isolated from a blood sample, labeled with the fluorescent dyes tetramethylrhodamine-ethyl-ester-perchlorate (TMRE) and MitoTracker Green FM (MTG) and then loaded onto a Live Cell Array slide. Images of cells in the array were taken and the relative fluorescence intensity of TMRE was used to measure the MMP. The same cells were imaged again after incubation with Oligomycin, an inhibitor of F1F0ATP synthase that hyperpolarizes the inner mitochondrial membrane. The distribution of individual cell intensities obtained for each patient was determined and comparisons were made for pre and post Oligomycin treatment. Preliminary results showed significant differences in MMP in patients compared to healthy controls, and following drug admission. The use of the Live Cell Array to study MMP in non-adherent cells at single cell level and cell populations offers insight into pathophysiology and variable cell responses in disease states.

2020/B484

High Throughput Flow Cytometry Screening of Cellular, Molecular, and Multiplexed Targets.

L. A. Sklar, C. Allen, B. S. Edwards, E. R. Prossnitz, M. Snider; University of New Mexico Center for Molecular Discovery, University of New Mexico, Albuquerque, NM

We have brought the power of flow cytometry to high throughput screening on behalf of the NIH Roadmap Molecular Libraries Probe Production Center Network. In collaboration with many investigative teams, we have conducted screens of phenotypic cellular targets (prostate cells, bacterial quorum sensing) and molecular targets (estrogen receptors, proteasome, VLA-4 integrin) as well as several families of multiplex targets including: 1) FPR family; 2) ABC transporters; 3) Bcl-2, 4) RGS, 5) small GTPases and 6) MMP substrate families. These screens reflect millions of data points uploaded to PubChem for >30 targets and have identified >10 novel probes. The GTPase family screens, for example, reveal both small molecule activators and inhibitors with intracellular activities. We are currently implementing molecular multiplex targets that include DNA and RNA protein interactions, kinase domain protein-protein interactions, and interactions of low molecular weight GTPases with their regulatory proteins. In cell-based high content analysis, we are exploring yeast multiplex model systems for TOR pathway analysis, cell cycle regulation, transporters, protein-DNA repair interactions, and host-pathogen yeast two hybrid protein interactions.

2021/B485

Genomic Stability Analysis of Immortalized Human Cells and Human Tumor Cells Using High-Resolution Array-Based Comparative Genomic Hybridization.

A. Kohara, M. Takeuchi, K. Takeuchi, Y. Ozawa, N. Hirayama, H. Mizusawa; National Institute of Biomedical Innovation, Osaka, Japan

INTRODUCTION: Genomic instability has been proposed to play an important role in cancer by accelerating the accumulation of genetic changes responsible for cancer cell evolution. Genomic aberrations in the form of subchromosomal DNA copy number changes including c-myc amplification are a hallmark of cell line. The goal of the present study was to analyze such aberrations in immortalized human cell lines and various tumor cell lines for regeneration medicine and scientific research at high resolution. METHODS: We employed high-resolution array comparative genomic hybridization with Agilent Human Genome CGH 244K and Affymetrix GeneChip Human Mapping 500K to analyze genomic aberrations in immortalized cell lines and various tumor cell lines. And also numerical chromosomal aberrations and rearrangements were analyzed by multicolor fluorescence in situ hybridization (mFISH). RESULTS: The immortalized human mesenchymal stem cell line UE6E7T-3 was shown to undergo a preferential loss of one copy of chromosome 13 after continuous culture. The loss of chromosome 13 was non-random and was detected by analysis of microsatellites and single-nucleotide polymorphism (SNP)-based loss of heterozygosity (LOH). Also, we have analyzed precise amplification units of c-myc genes in extra chromosomal double minute chromosome (DM) on tumor cell lines. These results suggested different patterns of c-myc amplification for DM generation in these cell lines. CONCLUSION: High resolution CGH analysis of immortalized cell lines and tumor cell lines reveals powerful tool for detection of chromosomal aberrations and gene amplification. In the regenerative medicine, the chromosome aberration is an important problem, and CGH might be useful a convenient and efficient screening method. Then, precise gene amplification analysis is important to elucidate a mechanism for oncogene amplifications for better understanding of tumor initiation or progression.

2022/B486

A Novel Strategy for the Cell Biology with the Fluoro-Laser Microdissection System (F-LMD).

N. Hachiya, K. Nishijima, K. Kaneko; Neurophysiology, Tokyo Medical University, Tokyo, Japan

Objective; Aggregation disorders mainly caused by the misfolded proteins include several human neurodegenerative diseases such as Alzheimer's disease, prion disease, and Huntington's disease. These aggregation-prone proteins, most likely in an oligomeric form, seem to play some roles in the development of these disorders, however, the basic mechanism that actually causes the neuronal cell death, has yet to be explained. With this background, we have developed a novel laser microdissection system (LMD) in combination with fluorescent-tagged proteins; fluoro-LMD (F-LMD). Our purpose is to elucidate the underlying molecular mechanisms by observing the behavior of the disease-associated proteins in living cells and providing the materials for further molecular analyses. Methods; (1) As the cell culture models for prion disease and Huntington's disease, mouse neuroblastoma Neuro2a cell lines expressing fluorescent-tagged proteins (GFP-prion protein (PrP), GFP-huntingtin (Htt)) were established. (2) F-LMD including a small material capture unit were developed, which enables us to dissect and collect the fluorescent proteins in living culture cells with no significant contamination of other subcellular components. Results and Conclusion; We are able to dissect and collect the fluorescent-labeled aggregates in the cells either expressing GFP-PrP or GFP-Htt. The F-LMD device can perform submicron order dissection and collection of the region of interest (<1μm square), which is now available for further proteomic analyses. Our novel biotechnology should allow us to combine morphology with biochemistry and cell biology at the molecular level.

2023/B487

Impedance-Based Measurements to Assess In Vitro Cytotoxicity.

J. Lo, D. Opp, C. Lo; Physics, University of South Florida, Tampa, FL

Among various cell-based assays, electrochemical impedance spectroscopy (EIS) using microelectrode arrays has emerged as a promising label-free method for detecting cellular responses to toxins of chemical or biological origin. However, in vitro assessment of cytotoxicity based on EIS needs more quantitative methods to analyze the alteration of cell morphology and motility, and hence the potential risk to human health. Here, we applied electric cell-substrate impedance sensing (ECIS) to evaluate dose-dependent responses of human umbilical vein endothelial cells exposed to cytochalasin B. To detect subtle changes in cell morphology, the frequency-dependent impedance data of the cell monolayer were measured and analyzed with a theoretical cell-electrode model. To detect the alternation of cell micromotion in response to cytochalasin B challenge, time-series impedance fluctuations of cell-covered electrodes were monitored and the values of power spectrum, variance, and variance of the increment were calculated to verify the difference. While a dose-dependent relationship was generally observed from the overall resistance of the cell monolayer, the analysis of frequency-dependent impedance and impedance fluctuations

distinguished cytochalasin B levels as low as 0.1 micromole. Our results show that cytochalasin B causes a decrease of junctional resistance between cells, an increase of membrane capacitance, and the reduction in micromotion. Thus, we report a strategic approach for ECIS and other EIS biosensors to investigate various aspects of cellular responses to toxins in general.

2024/B488

Real-Time Molecular-Level Assessment on Lipid Peroxidation of Single Cells Using Integrated Laser Tweezers and Raman Spectroscopy.

W. Chang[1], H. Lin[2], H. Chen[2], W. Chen[1], I. Liau[2]; [1]National Taiwan University Hospital, Taipei, Taiwan, [2]Institute of Molecular Science, National Chiao Tung University, Hsinchu, Taiwan

Background: Oxidative stress and the resultant lipid peroxidation are implicated in the pathogenesis of diverse diseases. Conventional assessment on lipid peroxidation depends mainly on measurements of the downstream products, thereby lacking in molecular details and temporal specificity. We propose that laser tweezers integrated with dynamic Raman spectroscopy can be used to real-time assess the lipid peroxidation of optically trapped single cell in a label-free manner. Methods: A laser (532 nm, 1.6 mW) was employed to capture a single yeast cell in medium and to produce Raman scattering of the trapped cell. Four spectral bands (1266, 1300, 1441, and 1651 cm-1) were identified and assigned to the =CH deformation, CH2 twisting, CH2 bending and C=C stretching modes, respectively. The dynamic changes of the Raman spectra were recorded upon different treatments. Similar measurements were also made on yeasts co-treated with ascorbic acid and simple liposomes comprising exclusively mono-unsaturated fatty acid 1-Palmitoyl-2-Oleoyl-sn-Glycero-3-Phosphocholine (POPC). Results: When the yeast was treated with hydroxyl radical, the Raman intensities at 1651 cm-1 ($P < 0.001$) and 1266 cm-1 ($P < 0.05$) declined gradually, while those at 1300 cm-1 and 1441 cm-1 remained unchanged (P = NS). The observation suggests that the C=C and =CH moieties decreased during the oxidative process, whereas that of the CH2 moieties remained unaltered, a result consistent with the oxidation of C=C bonds in lipids. Similar spectral changes in liposomes were obtained further supporting this notion. Also, the detection of increased lipid peroxidation product malondialdehyde, and reduced decrease in the Raman intensities by the co-treatment of ascorbic acid, again suggest that the dynamic spectral changes were indeed the result of lipid peroxidation induced by oxidative stress. Conclusion: We herein demonstrated a novel approach for real-time, label-free assessment on the lipid peroxidation of single cells. The same approach can be extended to studies focusing on the biochemical transformation of single cells or sub-cellular organelles under various stresses.

2025/B489

Micro-Array Cellchip Technology Is a Novel Tool for High-Throughput Adherent Single Cell Assays.

M. Kononov[1], J. Procopi[1], Y. Wang[2], C. Sims[1,2]; [1]Biology Research, Intellego Corp., Morrisville, NC, [2]Chemistry Research, Intellego Corp., Research Triangle Park, NC

Objecitve: Strategies for the selection, sorting and cloning of adherent cells in heterogeneous population are crucial for biological and biomedical research. Despite the significant progress in new method development, manipulation and collection of single viable cells remains an obstacle. Intellego Corp. is developing an innovative micro-array platform that combines state-of-the-art microfabrication technology with an integrated laser-microscope platform which enables a new class of single cell assays. We tested CellChip arrays with different adherent cell lines in several cell based assays such as cell sorting, viable cell separation and stable transfections. Methods: Mammalian and human adherent single cells or colonies were grown in the tissue culture media on the CellChip arrays composed of releasable “pallets” microfabricated from biocompatible polymer attached to glass substrate. The arrays were screened by microscopic imaging, and based on the assay type the specific individual cell/pallet were released, collected and cultured. Results: Morphology, cell growth and viability of six different mammalian and human cell lines were monitored throughout the isolation process. Single cells or colonies remain adherent during and after release for all cell lines tested. The released pallets were successfully collected with the efficiencies ranging from 55 -90%. Majority (up to 89%) of the cells remained viable and proliferated after cell/pallet collection. Using fluorescence marker, several independent transfection experiments on the CellChip resulted in rapid selection and isolation of stably transformed cell lines. Conclusion: The CellChip technology enables the robust adherently single cell screening and selection for a particular characteristic followed by efficient collection of the desired cells while the cells remain adherent to their growth surface.

2026/B490

Droplet Microfluidic Technology for Single-Cell High-throughput Screening.

E. Brouzes[1,2], M. Medkova[1], N. Savenelli[1], D. Marran[1], M. Twardowski[1], J. Rothberg[1], D. Link[1], N. Perrimon[2,3], M. Samuels[1]; [1]RainDance Technologies, Lexington, MA, [2]Genetics Department, Harvard Medical School, Boston, MA, [3]Howard Hughes Medical Institute, Boston, MA

We have developed a droplet microfluidic technology that enables single-cell high-throughput screening. Here we present the successful use of this quantitative on-chip droplet technology to screen an optically-encoded MitomycinC drug library for its cytotoxic effect against human monocytic U937 cells. The microfluidics platform permits the encapsulation of single-cells and reagents in aqueous droplets dispersed in a fluorocarbon oil carrier, providing physical and chemical isolation to each cell and allowing for their digital manipulation at very high-throughput. Compared to other microfluidics techniques, the droplet format eliminates risk of contamination, permits the thorough mixing of reagents and minimizes sample dispersion. In addition, this technology enables the analysis of small samples (i.e. primary cells, patient sample), consuming a low number of cells per condition. We show the encapsulation of U937 cells, their survival up to 4 days and the generation of MytomycinC dose-response curves. First, we developed an on-chip cytotoxicity assay to assess the viability of encapsulated cells. The assay integrates 5 modules: 1) 2 nozzles for encapsulating the cells and the dyes for the live-dead assay; 2) a fusion module to merge cells with the fluorescent dyes; 3) a mixing module; 4) an incubation line; and 5) a detection module. The assay was fully characterized and validated by accurately scoring different mixtures of live and dead cells. Second, we used this assay to optimize the cell encapsulation and storage conditions so that cell survival remains over 80% for a period of up to 4 days. Finally, to demonstrate the screening potential of the droplet technology, we screened an optically-encoded MitomycinC drug library for its cytotoxicity on U937 cells. The screen consisted of 3 steps: 1) an optically-encoded compound library was generated; 2) single cells were encapsulated and fused with the

compound library droplets; 3) after off-chip incubation, cell viability was scored and the optical scheme decoded on-chip. We show that we can optically resolve the different members while scoring for their cytotoxic effect, and generate a dose-response curve comparable to that found in a microtitre plate.

2027/B491

The Study of Erythrocyte Surface Potential Modifications Involving Malaria Parasite-Dependent Topographical Changes of Erythrocyte in Different Hosts.

E. Hayakawa[1], J. A. Dvorak[2], F. Tokumasu[2]; [1]International Research and Educational Institute for Integrated Medical Sciences(IREIIMS), Tokyo Women's Medical University, Tokyo, Japan, [2]LMVR, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD

Infection of erythrocytes by malaria parasite dramatically modifies host membranes both topography and internal physiological conditions. For example, 'knob' is accidented prominence and formed on erythrocyte surface after *Plasmodium falciparum*, a human malaria parasite, invasion, and some parasite derived proteins (PfEMP-1, KAHRP, PfEMP-3, etc.,) express in inner space of knob. As these results, erythrocyte membrane's physico-chemical properties are modified. Similarly, *P.gallinaceum*, a chicken malaria parasite are known to cause ridge-like structures on parasitized erythrocyte membrane. However, a rodent malaria parasite, *P.chabaudi*, does not form any knob-like structure, even parasite-derived proteins are expressed on erythrocyte membrane. Since parasite-derived proteins often responsible for the level of virulence, it is important to study the effect of those topographic modifications. To investigate erythrocyte surface modifications which is caused by malaria parasite in different hosts, we measured the zeta potential for parasitized- and unparasitized-erythrocytes. Zeta potential is assumed to be the electrochemical potential of cell surface. In *P.chabaudi*-infected mouse erythrocytes, zeta potential values of parasitized cells increased ~31% to negative direction when compared with that of uninfected cells. In contrast, *P.gallinaceum* which forms ridge-like structure on the host cells, the zeta potential decreased ~29% to positive direction. Similar trend of zeta potential shift was also observed in knob-forming *P.falciparum*-infected erythrocyte surface. These data suggested that in addition to the number and net charge of newly expressed parasite-derived proteins, topographic change induced by parasite infection may be an important factor to determine the direction of membrane potential modification.

2028/B492

Quantitative Characterization of Cardiomyocyte Contraction Using Scanning Probe Microscopy.

W. Chang[1], D. Yu[2], W. Chen[1], I. Liau[2]; [1]National Taiwan University Hospital, Taipei, Taiwan, [2]Institute of Molecular Science, National Chiao Tung University, Hsinchu, Taiwan

Background: Direct measurement on cardiomyocyte contraction is of great research interest. Scanning probe microscopy (SPM) has enabled the measurement of microscopic motion, and can potentially be applied to study cardiomyocyte contraction. Material and Methods: We sough to develop a system using SPM for characterizing the contraction of chick embryo cardiomyocytes. The piezo-controlled cantilever of the SPM was kept in contact with the cell membrane. The force of contraction exerted on the cantilever was maintained constant by dynamically adjusting the position of the cantilever. The motion of the membrane was then transformed into the displacement of the cantilever. The position of the micro-cantilever was recorded in real time to represent the contraction of the cardiomyocytes. Short-time Fourier transform was employed to analyze the data and produce a spectrogram that demonstrated the temporal changes of the amplitude and frequency of cardiomyocyte contraction. Results: Comparison of the spectrograms obtained on a confluent and a sub-confluent layers of cardiomyocytes showed that the contraction of confluent cardiomyocytes maintained at a constant amplitude and frequency for up to hours under a physiological condition (37oC, 5 % CO2), whereas the amplitude and frequency of contraction in the sub-confluent layer varied from time to time. The contractions under different pharmacological interventions have also been characterized. When the cardiomyocytes were treated with epinephrine, the amplitude and frequency of the contraction increased significantly. In contrast, esmolol significantly decreased both the amplitude and frequency of the contraction. Consistent with the stimulatory and inhibitory effects of beta-receptors on cardiomyocytes, this approach well demonstrated the ability of SPM to quantitatively assess the contractility as well as the contraction frequency of cardiomyocytes. Conclusion: Quantitative characterization of the amplitude and frequency of cardiomyocyte contraction has been delicately demonstrated using SPM. Through intuitive while quantitative graphic representation of these contraction characteristics, SPM can be applied in researches focusing on biomechanics in cell biology.

2029/B493

Proximity Ligation Assay (PLA) Correlates ANXA7 Protein Abundance with Genetic Status in Glioblastomas.

J. J. Renfrow[1], A. K. Yadav[1], H. Vogel[2], G. R. Harsh[2], J. P. Chandler[1], M. Bredel[1,2]; [1]Northwestern University, Chicago, IL, [2]Stanford University, Stanford, CA

Background: Our previous functional and clinical investigations implicate *ANXA7* as a candidate tumor suppressor on chromosome 10q21.1-q21.2 in human brain tumors, which is deleted in about 50% of glioblastoma multiforme and might function as a haploinsufficiency gene. In addition to gene dosage effect on transcription, *ANXA7* deletion might lead to decreased ANXA7 protein abundance. Proximity Ligation Assay (PLA) is a novel highly sensitive and specific, quantitative protein technology capable of visualizing single protein molecules in formalin-fixed, paraffin-embedded tissue. In this study we examined the utility of PLA to demark hemizygous *ANXA7* losses at the protein level. **Methods:** A cohort of twelve archived glioblastomas from Stanford University was used in this study. The tumors' genetic status for *ANXA7* was determined through microarray-based comparative genomic hybridization (array-CGH) analysis. Corresponding protein abundance was determined in a blinded fashion utilizing PLA. PLA amplifies the specificity of antibody recognition by using a proximity probe of antibodies carrying tethered nucleic acid sequences that are amplified *in situ* and then detected with a complimentary fluorescently labeled oligonucleotide. Slides were analyzed using fluorescent microscopy and BlobFinder software to quantitate ANXA7 abundance. **Results:** ANXA7 protein abundance ranged from 0.089 signals/cell to 11.22 signals/cell. The group of *ANXA7* deleted tumors' average protein abundance was 0.365 signals/cell while the wild-type group averaged 5.247 signals/cell. PLA revealed GBM tumors wild-type for *ANXA7* were reliably differentiated to have higher protein expression than *ANXA7*-deleted tumors (p=0.004, unpaired Student's t-test).

Protein abundance for ANXA7 thus mimics the genetic level status. **Conclusions:** *ANXA7* deletion in glioblastomas leads to a corresponding loss of protein expression in these tumors, which ultimately impacts tumor behavior. PLA is a tool well suited to assess hemizygous and homozygous gene deletions in formalin-fixed, paraffin-embedded tissue.

2030/B494

Sorting and Expansion of Murine Embryonic Stem Cell Colonies Using Micropallet Arrays.

H. Shadpour, C. Sims, R. Thresher, N. Allbritton; University of North Carolina, Chapel Hill, NC

The goal of this work was to develop an efficient method to isolate undifferentiated stem cells from differentiated cells. Conventional techniques for colony selection and isolation require significant time, labor, and consumption of expensive reagents. New microengineered technologies hold the promise for improving colony manipulation by enhancing the efficiency of colony picking and reducing the required manpower and reagent consumption. Murine embryonic stem cells were cultured on arrays composed of releasable elements termed micropallets created from a biocompatible photoresist. Micropallets containing undifferentiated colonies were released using a laser-based technique followed by cell collection and expansion in culture. The micropallet arrays provided a biocompatible substrate for maintaining undifferentiated murine stem cells during colony formation in culture. A surface coating of 0.025% gelatin was shown to be optimal for cell culture and collection. Arrays composed of micropallets whose surfaces had been modified on the nanometer scale provided further improvements in culture and isolation. Using this platform, colonies of viable stem cells were efficiently isolated and collected. Colonies sorted in this manner were shown to remain undifferentiated even after collection and further expansion in culture. Qualitative and quantitative analyses of sorting, collection efficiency and cell viability after release and expansion of stem cell colonies demonstrate that the micropallet array technology is a promising alternative to conventional sorting methods for stem cell applications.

2031/B495

Enrichment and Expansion of Cells Using Antibody-Coated Micropallet Arrays.

C. Sims, H. Shadpour, N. Allbritton; Chemistry, University of North Carolina, Chapel Hill, NC

Positive selection, sorting, and collection of single cells from within a heterogeneous population are steps required for many cell-related biological studies. We recently demonstrated a miniaturized cell array for positive selection and collection of single cells. In the current work, the combination of a surface antibody enrichment step with this microarray technology is shown to be an efficient method for isolating specific target cells from within a mixed cell population. Mixtures of target and non-target cells with initial populations of fewer than 20,000 target cells/mL were used for sorting studies using antibody-coated microarrays to pre-enrich the target population. Antibodies directed against the cell surface receptors Fc(epsilon)R1, c-Kit, and ErbB2 were used to perform positive selection of RBL, RBL, and SK-BR-3 cells, respectively, from a mixed population containing the target cell admixed with 3T3 and HeLa cells, two cell types that lacked the specific ligands. The capture efficiency, selectivity, and enrichment for the target cells were calculated and compared to fibronectin-coated control micropallet arrays. As expected, the capture efficiency depended upon the frequency of the target cell in the mixed population over the range of 0.3% to 33%. For a frequency of 5% target cells, the capture efficiency was 27 - 36% for the three conditions, while the selectivity varied between 71 - 96% with a 14 - 19-fold enrichment. Single-cell cloning studies demonstrated a high cloning efficiency of target cells selected from the micropallet array. The data indicate that antibody pre-enrichemnt should prove valuable in combination with micropallet-based cell selection for sorting rare cells from a heterogeneous population.

2032/B496

Use of FTIR to Assess the Cellular Response of Saccharomyces Cerevisiae to the Presence of Heavy Metals.

P. Chin[1], R. Kodoru[1], R. Wang[2], G. Hastings[2], J. K. Hilliard[1], J. E. Houghton[1]; [1]Biology, Georgia State University, Atlanta, GA, [2]Physics & Astronomy, Georgia State University, Atlanta, GA

Fourier Transform Infrared Spectroscopy (FTIR) potentially provides a nondestructive and direct way to detect the metabolite changes inside the cell. A wide spectrum of IR can be absorbed by the chemical bonds of metabolites inside the cell. In this work FTIR technology is being applied to investigate the immediate cellular response and physiological changes in Saccharomyces cerevisiae that result from exposure of the cells to heavy metals. The potential of FTIR to assess such changes includes the potential to analyze changes that result from the apoptotic program that we have recently demonstrated is part of the cellular response to the presence of heavy metals. In attempting to evaluate these cellular changes we also show some of the more recent adaptations in infrared microspectroscopy to detect the molecular/metabolic changes in the apoptotic response of wild-type and non apoptotic yca1Δ mutant cell lines. Sample preparation protocols and preliminary a study of Cd-induced apoptotic cell for IR spectrometry are shown in this study, and some of the more notable changes in resultant IR spectra evaluated. In an attempt to analyze further the changes in absorption vibration that are due to the actual cellular responses to apoptosis, but not temporal effects on growth of the culture, appropriate control experiments were undertaken on wild-type and non apoptotic, yca1Δ mutant cell lines. Furthermore, large sample sizes and increased number of independent sample sources ($N > 8$) were used so as to enhance evaluation of parameters that are directly effected by the presence of heavy metals in the medium. Given the ready availability of various deletion libraries for yeast, the findings presented in this study suggest that IR microspectroscopy may prove to be a highly useful tool in studying metabolic changes in yeast cells undergoing metal-induced oxidative stress and/or apoptosis.

2033/B497

Monitoring Dynamic Cellular Events by Rapidly Regulating the Amount of a Protein via a Membrane Permeant Ligand.

M. Haugwitz, T. Garachtchenko, O. Nourzaie, S. Gandlur, C. Mello, T. Quinn, H. Sagawa; Clontech Laboratories, Inc., Mountain View, CA

The ability to quickly change the amount of a specific protein in live cells allows one to monitor dynamic events in a cell. Traditionally, the amount of a protein can be regulated either at the transcriptional level via inducible promoters or knockdown approaches using homologous recombination, or at the translational level using RNAi. However, these approaches target upstream events and do not change the amount of the protein of interest directly, on a post-translational level. Therefore, they are not applicable to studies that seek to reveal a protein's function and dynamic behaviors by quickly changing its cellular abundance. Recently, a 12 kDa (107 amino acid) mutant of the FKBP

protein (the destabilization domain, or DD as part of the ProteoTuner™ system) was reported to destabilize the protein it is fused to, by targeting the fusion protein to the proteasomes. However, when the small (750 Da) membrane permeant ligand, Shield1, is added to the cells' culture medium, it binds to the DD and protects the fusion protein from proteasomal degradation. This causes a rapid accumulation of the protein inside the cell. Varying the concentration of Shield1 that is added to the culture allows one to fine-tune the extent of stabilization. This is a reversible process, meaning that removing Shield1 from the cells causes the DD-tagged protein to be degraded very quickly, with a $T_{1/2}$ of ~1 to 2 hours. We took advantage of the system's rapid kinetics to monitor the dynamics of actin filament turnover. It enabled us to determine that the actin filament system rearranges very rapidly, in a matter of only 15-30 minutes. We also used the system to successfully rescue the ability of a ZAP70-negative cell line to secrete IL2 upon stimulation by reintroducing DD-ZAP70 in the presence of Shield1. In order to monitor these rapid events in the cell, it was crucial to use a method that provided rapid control of the protein of interest. Methods that work at the transcriptional or translational levels would not have been sufficient for these studies.

2034/B498

Comparison of Nucleic Acid Extraction Methods for Removal of PCR Inhibitors from Environmental Samples Including Soil and Plants.

W. Kim[1], Y. Haj-Ahmad[2,1]; [1]Norgen Biotek Corp., Thorold, ON, Canada, [2]Brock University, Thorold, ON, Canada

Direct lysis techniques have proven extremely useful for studying the nucleic acids of microbial communities of soils and plants because they avoid the traditional and time-consuming culture-based microbiological methods. However the major drawback with direct lysis techniques is that large amounts of PCR-inhibitory substances are extracted along with the DNA. These inhibitors interfere with the cell lysis step, inactivate the thermostable DNA polymerase and interfere with nucleic acids. A number of different techniques are used to remove PCR inhibitory substances, and the most common is the incorporation of PCR facilitators directly in the PCR reaction. However, a more efficient method to deal with the inhibitors would be to exclude their co-purification during nucleic acid extraction. Thus, in this study we tested and compared a number of different nucleic acid extraction methods for isolating DNA from soil and grape samples, and we found that there is a significant difference between these methods for removing PCR inhibitors. Here we report on the effectiveness of various nucleic acid extraction kits for PCR inhibitor removal from different soil and grape samples.

2034A/B499

Live-Cell Imaging: Characterizing and Moderating Photodamage.

S. Galdeen, G. Sluder; Cell Biology, University of Massachusetts Medical School, Worcester, MA

Phototoxicity is a serious and limiting problem in fluorescence time-lapse studies of live cells. We use normal human somatic cells to characterize the phototoxic effects of excitation wavelengths commonly used in fluorescent protein microscopy. We find that excitation wavelengths used for GFP (480-500 nm) cause a durable G1 arrest in 50% of the cell population after ~20s at 140 $\mu W/cm^2$. Wavelengths used for excitation of red fluors such as RFP and mCherry (535-555 nm) are only slightly better (50% arrest at ~30s exposure). However, the spectra used for emerging far-red fluorophores (610-630 nm) is significantly less damaging, with continuous exposure times of up to 3 minutes having no effect on the cell cycle. These results suggest that a transition to far-red fluorophores may be one way to increase viability for live-cell timelapse studies. We are investigating how to moderate phototoxic effects by inhibiting the p38 stress-activated kinase and by eliminating leakage of long-wave UV through excitation filters.

Mechanism of Nuclear Transcription (2035 – 2056)

2035/B500

Light Activation of Single Genes Provides a Quantitative Basis for Measuring Transcription.

D. Larson[1,3], S. Nandy[1], D. Lawrence[2], J. Condeelis[1,3], R. Singer[1,3]; [1]Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY, [2]Chemistry, University of North Carolina, Chapel Hill, NC, [3]Gruss-Lipper Biophotonics Center, Albert Einstein College of Medicine, Bronx, NY

Transcription is regulated to achieve specific levels of mRNA in specific tissues at precise times. Although many of the molecular components of transcription regulation are known, very little is known about the kinetic regulation of single genes in living cells. For example, it is unknown whether or not multiple transcripts can be initiated from a single initiation complex. Furthermore, little is known about putative transcriptional pause states or the possible significance of pausing in vivo. We have developed a light-activated gene expression system that allows transcription to be intiated with temporal and spatial control. The light activated gene is based on the ecdysteroid expression system and consists of genetically encoded nuclear receptors, a reporter gene containing the response element, and ectopically applied caged ecdysone which becomes active upon UV photolysis. Using the phage MS2 coat protein to selectively bind mRNA stem loops in the reporter gene, we can visualize each stage of expression of single genes in living cells, proceeding from nascent RNA at a nuclear transcription site, to individual mRNA in the cytosol, and finally to protein. We find that transient stimulation results in accumulation of nascent mRNA at the site of transcription. These complexes are not transcriptionally active as demonstrated by photobleaching recovery. Under certain conditions, we are able to see the initiation of transcripts in discrete steps corresponding to single transcripts. By precisely controlling the dose of active ecdysteroid with light - both in time and amplitude - we can achieve maximal levels of protein expression. These data suggest that the kinetic sequence of activating ligand is important in initiating and maintaining transcription. In addition, transcriptional pausing may be a control mechanism which regulates transcription downstream of the transcription start site. The ability to control gene expression with spatial and temporal precision thus requires a quantitative mechanistic understanding of how single genes are regulated in cells. Exploratory Centers for High Resolution Imaging Probes, 1P20EB004930, NIH. Cell Migration Consortium Grant No, U54 GM064346 from NIGMS.

2036/B501

Real Time Transcription of an Endogenous Gene.

T. Lionnet[1], J. Chao[1], Y. Shav-Tal[2], X. Darzacq[3], R. Singer[1]; [1]Anatomy & Structural Biology, Albert Einstein College of Medicine, Bronx, NY, [2]The Mina & Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat-Gan, Israel, [3]CNRS - UMR 8541, Ecole Normale Superieure, Paris, France

In order to ensure stable expression of an endogenous protein, the cell has to regulate each step of gene expression, starting with transcription. How transcription is temporally regulated is still an open question. Here we observe in live cells the real-time transcription of an endogeneous gene, β-actin. To achieve this, we use a transgenic mouse model where RNA stem loops are knocked in the untranslated region of β-actin mRNA, and when expressed are bound to a fluorescent MS2 reporter protein. We monitor β-actin transcription levels in single fibroblast cells, either as a response to serum induction or in conditions of basal expression (constant serum level). This way we are able to describe the kinetics of β-actin transcription over a long period. We observe that the cell responds to serum induction within minutes, and that transcription falls back to basal levels after ~1h, in agreement with data on fixed cells. We then compare how levels of transcription vary within one cell (between the alleles), and from cell to cell within a cell population. These findings uncover how extrinsic causes (influence of the environment within the cell) and intrinsic causes (inherent stochasticity of transcription at one gene locus) respectively contribute to genetic expression noise of an essential endogeneous gene.

2037/B502

Sp1 and Sp3 Regulate Transcription of the Human Glycolipid Transfer Protein (GLTP) Gene.

X. Zou, Y. Gao, H. M. Pike, R. Brown; Hormel Institute, Austin, MN

Glycolipid transfer protein (GLTP) is a small (23-24 kDa) cytoplasmic protein that accelerates the intermembrane transfer of various glycolipids and is highly conserved in vertebrates. Expression profiles suggest that GLTP plays a fundamentally important role in eukaryotic cells. To gain new insights into the expression of this protein, we cloned and characterized the promoter region of human GLTP gene to evaluate its transcriptional regulation. Luciferase reporter assays demonstrated that a 1169bp GLTP promoter sequence (from -1150 to +19 relative to the major transcriptional start site) displays constitutive promoter activity. A series of 5' deletion and 3' deletion mutants of the 5' flanking region, cloned into pGL3-Basic luciferase plasmids, enabled identification of a 225bp region (from -350 to -126) as the GLTP basal promoter by luciferase reporter assays. A negative regulatory region that down-regulates GLTP promoter activity was found upstream of -416. Using electrophoresis mobility shift assays and chromatin immunoprecipitation assays, we demonstrated that Sp1 and Sp3 can bind to multiple, specific GC-box sites. Mutational analysis confirmed that the activity of the GLTP basal promoter depends on the presence of functional Sp1 and Sp3 binding sites and showed that mutation of one particular GC-box site is especially detrimental to promoter activity. Overexpression of the Sp1 and Sp3 transcription factors also was found to reduce GLTP promoter activity. Altogether, these results provide the first characterization of the human GLTP promoter and identify Sp1 and Sp3 as important transcriptional regulators of the human GLTP gene. [Support: NIH GM45928, NIH CA121493, the Hormel Foundation].

2038/B503

The 19S Atpase S6a Regulates Transcription of the Critical Immune Response Factor CIITA.

A. D. Truax[1], O. I. Koues[2], S. F. Greer[1]; [1]Division of Cell and Molecular Biology and Physiology, Department of Biology, Georgia State University, Atlanta, GA, [2]Division of Molecular Genetics and Biochemistry, Department of Biology, Georgia State University, Atlanta, GA

The 26S proteasome is composed of a 19S regulatory particle which recognizes and unfolds polyubiquitinated proteins and a 20S proteolytic core in which the unfolded proteins are degraded. Recent evidence supports a novel, non-degradative role for components of the 19S regulator as mediators of transcriptional systems in yeast. The 19S contains six ATPases which have been demonstrated to associate with actively transcribed genes and to be required for transcription from multiple yeast promoters. We have recently shown that in mammalian cells the Major Histocompatibility Class II (MHC-II) *HLA-DRA* promoter is similarly regulated by actions of the 19S ATPases. MHC-II molecules are glycoproteins that present extracellular antigens to CD4+ T cells and play an important role in the induction and regulation of adaptive immune responses. MHC-II molecules are regulated at the level of transcription by a master regulator, the class II transcriptional activator (CIITA) whose association with the MHC-II promoter is absolutely necessary to initiate MHC-II transcription. Here, we have investigated the role of the 19S ATPase S6a in regulating CIITA mediated MHC-II transcription. In the presence of inflammatory cytokines, S6a is recruited to the inducible CIITA promoter, pIV. siRNA mediated S6a knockdown correlates with a loss of transcription initiation at CIITA pIV and markedly decreased CIITA expression. Histone H3 and H4 acetylation and the recruitment of transcription factors necessary for initiating inducible CIITA expression are blocked in the absence of S6a; indicating a role for S6a and the 19S ATPases in regulating epigenetics and the opening of closed chromatin at CIITA pIV. These observations support a novel, degradation independent role for 19S ATPases in regulating mammalian transcription and present the proteasome as a potential therapeutic target for manipulating MHC-II expression and the adaptive immune response. Research supported by the NMSS, The Georgia Cancer Coalition, and The Molecular Basis of Disease Area of Focus at GSU.

2039/B504

What Can Brown Do for You? The Many Roles of the Ubiquitin-Proteasome System (UPS) and Monoubiquitination in Regulating Mammalian Transcription.

K. P. Bhat, J. K. Brooks, S. F. Greer; Cellular and Molecular Biology and Physiology, Department of Biology, Georgia State University, Atlanta, GA

Major histocompatibility class II (MHC II) molecules are glycoproteins that present extracellular antigens to CD4+ T cells and are essential for the initiation of adaptive immune responses. MHC II expression is regulated at the level of transcription and requires recruitment of a master regulator, the class II transactivator (CIITA), to the MHC II promoter. We have previously linked CIITA to the UPS by demonstrating that monoubiquitination of CIITA dramatically increases its transactivity whereas polyubiquitination leads to CIITA degradation. The 26S proteasome, itself a master regulator of protein degradation, is composed of a 19S regulatory particle which recognizes and unfolds

polyubiquitinated proteins and a 20S proteolytic core in which the unfolded proteins are degraded. The 19S regulator contains 6 ATPases which have been shown to be required for transcription of several yeast genes. We have recently demonstrated that the 19S ATPase Sug1 is inducibly recruited to the MHC II promoter, is required for activating epigenetic modifications at the promoter and is necessary for stable binding of CIITA to the promoter; all suggesting a novel, non-proteolytic role of the 19S in regulating MHC II gene transcription. Here, to determine the mechanisms regulating the interactions of CIITA and Sug1, extensive mapping experiments were performed. We show that Sug1 binds to the CIITA amino terminal degron sequence within aa 226-335 of CIITA. The association of Sug1 with CIITA is further enhanced by CIITA monoubiquitination on proximal lysine residues. Mutation of these lysine residues results in a loss of CIITA transactivation, indicating that it is monoubiquitination of CIITA, proximal to the degron sequence, which leads to association with the 19S proteasome and stable binding of CIITA to the MHC II promoter. Because much remains to be known of the non-proteolytic role of UPS in mammalian gene transcription, these studies will both broaden our understanding of the ways in which the UPS regulates mammalian gene transcription and will provide novel strategies for manipulating the expression of MHC II genes. Research supported by NMSS, the GCC and GSU MBDAF.

2040/B505

Adaptive Cellular Glucose Response Involves the Mondoa: Mlx Transcription Factor Complex and Its Direct Target Gene, TXNIP.

C. A. Stoltzman, D. E. Ayer; Oncological Sciences, Huntsman Cancer Institute, Salt Lake City, UT

Glucose is a fundamental metabolite, yet how cells sense and respond to physiological changes in glucose concentration is not completely understood. Here we consider the role of the MondoA:Mlx heterodimeric transcription factor in sensing, responding to and regulating intracellular glucose levels. In their latent state, MondoA:Mlx complexes localize to the outer mitochondrial membrane, yet shuttle between the mitochondria and the nucleus. We show that MondoA:Mlx complexes accumulate in the nucleus in response to glucose and its non-metabolizable analog 2-deoxyglucose (2DG) in vitro. Lack of nuclear MondoA:Mlx accumulation in cells treated with 3-bromopyruvate or non-phosphorylatable glucose analogs further demonstrates that nuclear localization of MondoA:Mlx depends on the enzymatic activity of hexokinases, which catalyze conversion of glucose to glucose-6-phosphate (G6P) in the first step of the glycolytic pathway. Together, these findings suggest that MondoA:Mlx monitors intracellular G6P concentration and translocates to the nucleus when levels of this key metabolite increase. Thioredoxin-interacting protein (TXNIP) was identified through these experiments as a direct and glucose-regulated MondoA:Mlx transcriptional target. MondoA:Mlx complexes act as potent negative regulators of glucose uptake and glycolysis via their upregulation of TXNIP protein levels, as well as through a TXNIP-independent mechanism as seen in TXNIP knockout mouse embryonic fibroblasts. These studies suggest a key role for MondoA:Mlx transcription factor complexes in the adaptive cellular response to changes in extracellular glucose concentration and peripheral glucose uptake.

2041/B506

Subcellular Shuttling of the Mondoa Transcriptional Activator.

C. Peterson, D. E. Ayer; Oncological Sciences, Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

Proper regulation of energy homeostasis is an essential requirement for all life forms. MondoA is a basic helix-loop-helix leucine zipper transcription factor that heterodimerizes with Max-Like factor X (Mlx) and localizes to the outside of mitochondria. The heterodimer shuttles between mitochondria and the nucleus, facilitating communication between these two essential organelles. MondoA:Mlx complexes accumulate in the nucleus to occupy the promoters of target genes in response to high glucose. Global gene expression experiments with a non-metabolizable glucose analog demonstrate that more than 75% of glucose-induced transcription depends on MondoA, suggesting a prominent role in energy homeostasis. Using a mammalian cell culture model system, here we probe the molecular mechanisms that control nuclear import, nuclear export and transcriptional activity of MondoA:Mlx complexes and how they are altered by high glucose to allow nuclear accumulation and activity. We assess MondoA:Mlx function by subcellular localization studies, transcriptional reporter assays, and chromatin immunoprecipitation. Results show that regions throughout both proteins are essential for various steps in the nuclear translocation cycle, including nuclear import via MondoA:Mlx C-terminal interactions, and nuclear export via Crm1 binding at the N-terminus of MondoA. Importantly, mutants have been isolated that constitutively reside in the nucleus yet do not activate transcription, suggesting that localization and activity of MondoA:Mlx are separable. MondoA mutations that disrupt 14-3-3 binding are constitutively cytoplasmic. Surprisingly, other mutations within the same conserved 14-3-3 binding region show similar localization phenotypes yet retain 14-3-3 binding, suggesting pleiotropic function of the MondoA 14-3-3 binding domain. We conclude that the response of MondoA:Mlx to intracellular glucose levels involves regulation of multiple steps of subcellular shuttling and diverse regions of each protein. Current work focuses on understanding the mechanisms of these structural controls and the identification of novel cofactors involved in the process.

2042/B507

The Dynamics of Single Mrnp Nucleo-Cytoplasmic Transport through the Nuclear Pore in Living Cells.

A. Mor, S. Suliman, Y. Shav-Tal; The Mina & Everard Goodman Faculty of Life Sciences, Bar-Ilan University, Ramat Gan, Israel

Transcribed mRNAs are released from the transcription site and travel through the nucleoplasm en route to the nuclear pores (1). The in vivo kinetics of mRNP export on the single mRNA level are uncharacterized. We generated a human cell system that allowed us to follow in vivo as single mRNPs traveled through the nucleoplasm, and translocated through the nuclear pore to emerge in the cytoplasm. In order to detect real-time single mRNP export we generated several gene constructs that transcribed large mRNAs, based on different versions of the large human dystrophin mRNA (11kb). These genes were under the transcriptional control of the ecdysone-inducible system. In order to detect the mRNPs in single living cells, the 3'UTR of the mRNAs contained a series of 24 MS2 repeats. These repeats were bound by YFP-MS2 proteins to yield tagged mRNP complexes, which were detected using live-cell fluorescence microscopy. Using rapid imaging, deconvolution and tracking techniques we could follow single mRNPs as they reached the nuclear envelope, unfolded, traveled through the pore, and restructured on the cytoplasmic side. We quantified the kinetics of single mRNP export as well as the diffusion properties of the traveling mRNPs before and after export. In addition, we examined these kinetic properties with respect to the presence or lack of an intron within the mRNA transcripts. Our novel data show that mRNA average travel time from the site of transcription to the nuclear envelope occurs within

10-20 minutes, while only few seconds are required for the rapid processes of mRNP restructuring and export. (1) Shav-Tal Y. Darzacq X, Shenoy SM, Fusco D, Janicki SM, Spector DL, Singer RH. Dynamics of single mRNPs in nuclei of living cells. Science 2004, 304:1797.

2043/B508

Regulation of P53 Target Gene Expression by Peptidylarginine Deiminase 4.

P. Li, H. Yao, D. Gilmour, Y. Wang; Center for Gene Regulation, Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA

The p53 tumor suppressor is a DNA-binding transcriptional factor that recruits both coactivators and corepressors to regulate the expression of its target genes. Histone Arg methylation has been correlated with transcriptional activation of p53 target genes. However, whether this modification is reversed to repress the expression of p53 target genes remains unknown. Here, we report that peptidylarginine deiminase 4 (PAD4), a histone citrullination enzyme, is involved in the repression of p53 target genes. Inhibition by PAD4 inhibitor Cl-amidine or depletion using PAD4 siRNAs elevated the expression of a subset of p53 target genes, including p21/CIP1/WAF1, leading to cell cycle arrest and apoptosis. Moreover, the induction of p21 expression, cell cycle arrest, and apoptosis by PAD4 depletion is p53-dependent. Protein-protein interaction studies showed an interaction between p53 and PAD4. Chromatin immunoprecipitation assays showed that PAD4 is recruited to the p21 promoter in a p53 dependent manner. RNA Polymerase II (Pol II) activities and the association of PAD4 are dynamically regulated at the p21 promoter during UV irradiation. Paused RNA Pol II and high levels of PAD4 were detected before UV treatment. At early time points after UV treatment, an increase of histone Arg methylation and a decrease of citrullination were correlated with transient activation of p21. At later times after UV irradiation, a loss of RNA Pol II and an increase of PAD4 were detected at the p21 promoter. The dynamics of RNA Pol II activities after UV treatment were further corroborated by permanganate footprinting. Together, these results suggest that PAD4 plays a role in the regulation of p53 target gene expression.

2044/B509

A Temperature-Sensitive Mutation of a WD Repeat-Containing Protein, Smu1, and Its Conserved Role in Splicing.

K. Sugaya, Y. Ishihara, K. Sugaya; Radiation Effect Mechanisms Research Group, National Institute of Radiological Sciences, Chiba, Japan

A temperature-sensitive CHO-K1 mutant cell line, tsTM18, exhibits chromosomal instability and cell cycle arrest at S and G_2 phases with decreased DNA synthesis at the nonpermissive temperature, 39°C. We previously identified an amino acid substitution in Smu1 that underlies the temperature-sensitive phenotypes of tsTM18 cells. Because genetic studies of nematodes revealed that *smu-1* is involved in splicing of the *unc52/perlecan* pre-mRNA, we analyzed the *perlecan* transcript in tsTM18 cells by reverse transcription-polymerase chain reaction (RT-PCR). The *perlecan* PCR product amplified from RNA of tsTM18 cells cultured at 39°C appeared to be a mixture of variants. Sequence analysis identified at least six variants that result from alternative splicing and intron retention. Comparison of the results of *perlecan* RT-PCR analysis with those of analysis of four other genes suggested that the splicing defect in the *perlecan* gene may be unique and that it is conserved through evolution. SF2/ASF is a member of the SR splicing factors, which are proteins necessary for an early step in spliceosome assembly and that can influence the selection of alternative splice sites. In tsTM18 cells cultured at the nonpermissive temperature, the temperature-sensitive defect of Smu1 appears to alter localization of SF2/ASF, suggesting that there is a link between Smu1 and SF2/ASF.

2045/B510

Requirement of N-Linked Glycosylation in the Luminal Domain for Optimal Proteolytic Activation of Liver-Enriched Transcription Factor CREB-H.

C. Chan[1], T. Mak[1], K. Chin[2], D. Jin[1]; [1]Department of Biochemistry, The University of Hong Kong, Hong Kong, China, [2]The Kimmel Center for Biology and Medicine, Skirball Institute, New York University School of Medicine, New York, NY

CREB-H is a liver-enriched bZIP transcription factor closely related to CREB3. CREB-H is activated by intramembrane proteolysis that releases an N-terminal fragment. Aberrant expression of CREB-H is implicated in hepatocellular carcinoma. In this study we characterized N-linked glycosylation of CREB-H which occurs at three sites in the C-terminal luminal domain. When all three sites were disrupted by site-directed mutagenesis, N-linked glycosylation of CREB-H was completely abrogated. The CREB-H mutants defective for N-linked glycosylation poorly activated transcription driven by unfolded protein response element or C-reactive protein promoter. Upon stimulation with an activator of intramembrane proteolysis such as brefeldin A, unglycosylated CREB-H remained largely uncleaved, anchored to the endoplasmic reticulum membrane, and unable to activate transcription. Taken together, our findings suggest that N-linked glycosylation is necessary for full activation of CREB-H through intramembrane proteolysis. Our work also reveals a novel mechanism for post-translational regulation of the CREB3 subfamily of membrane-associated transcription factors.

2046/B511

Hyperglycemia Mediates Down-Regulation of Angiotensin Type 1 Receptor through Alterations in HDAC Bioavailability.

P. G. Biju, R. Yesudas, T. Thekkumkara; Department of Pharmaceutical Sciences, Texas Tech University Health Sciences Center, 1300 S Coulter, Amarillo, TX

The angiotensin type I receptors (AT1R) belongs to the class of seven transmembrane receptors that couple to G-proteins. Previously, we identified the cis-acting element of glucose-mediated AT1R transcriptional repression. In the present study, we used a continuously passaged rat liver epithelial cell expressing native AT1R to determine the cellular mechanism(s) for glucose-mediated AT1R transcriptional repression. The cells were exposed to 5.5 mM normal glucose (NG) and 25 mM high glucose (HG) for 48 h and changes in Angiotensin II (Ang II) binding, and AT1R protein and mRNA expressions were assessed. HG exposed cells exhibited significant down-regulation of AngII specific binding (52108 ± 1052 to 31360 ± 1626 dpm/mg protein), and AT1R protein and mRNA expression. In addition, we observed significant activation of protein kinase C (PKC) and mitogen-activated protein (MAP) Kinase in HG exposed cells. Under similar conditions, highly selective cell permeable PKC inhibitor bisindolylmaleimide eliminated HG mediated MAP-Kinase activation and AT1R down-regulation. Similarly MAP-Kinase Kinase (MEK) inhibitor PD 98059 prevented HG mediated AT1R down-regulation. Furthermore, treatment of HG

exposed cells with Trichostatin A (HDAC class I/II inhibitor) reversed AT1R repression, suggesting that the catalytic and nuclear bioavailability of HDACs may play a role in HG induced AT1R repression. Our results show for the first time that a PKC activated MAP-Kinase signaling pathway induced chromatin modification machinery is responsible for the AT1R gene repression in hyperglycemia. Since AT1R plays a critical role in the control of hypertension, glucose-induced changes in receptor expression may participate in diabetes-associated complications (Supported by NIH Grant DK072140).

2047/B512

Glutamate Regulation of NF-Kappab-dependent Gene Expression.

R. Sitcheran, W. C. Comb, P. C. Cogswell, A. S. Baldwin; Lineberger Comprehensive Cancer Center, UNC at Chapel Hill, Chapel Hill, NC

Glutamate is a critical neurotransmitter of the central nervous system (CNS) and also an important regulator of cell survival and proliferation. Binding of glutamate to metabotropic glutamate receptors (mGluRs) induces signal transduction cascades that lead to gene-specific transcription. The transcription factor NF-kappaB, which regulates cell proliferation and survival, is activated by glutamate; however, the glutamate receptor-induced signaling pathways that lead to this activation are not clearly defined. Here we investigate glutamate-induced activation of NF-kappaB in glial cells of the CNS, including primary astrocytes. We show that glutamate/mGluR5 stimulation induces phosphorylation, nuclear accumulation, DNA binding, and transcriptional activation function of glial p65. Glutamate-induced activation of NF-kappaB requires calcium-dependent IKKalpha and IKKbeta activation and induces p65-IkappaBalpha dissociation in the absence of IkappaBalpha phosphorylation or degradation. Moreover, glutamate-induced IKK preferentially targets phosphorylation of p65, but not IkappaBalpha, and the ability of glutamate to activate NF-kappaB requires cross-coupled signaling with the epidermal growth factor receptor (EGFR). Finally, we show that these distinct signaling events lead to differential regulation of NF-kappaB-dependent gene expression and control of cell proliferation/viability. Our results provide insight into a glutamate-induced regulatory pathway distinct from that described for cytokine-induced NF-kappaB activation and highlight the remarkable specificity of NF-κB activity to control gene expression in response to diverse stimuli.

2048/B513

Selective Activation of NFAT by Promyelocytic Leukemia Protein.

Y. Lo[1,3], C. Wu[1], H. Shih[2], M. Lai[1,3]; [1]Molecular biology, Academia Sinica, Taipei, Taiwan, [2]Biomedical Sciences, Academia Sinica, Taipei, Taiwan, [3]Microbiology and Immunology, National Yang-Ming University, Taipei, Taiwan

Promyelocytic leukemia (PML) protein is a tumor suppressor. It is involved in many different physiological functions but the complicated action mechanisms are not yet fully understood. Nuclear factor of activated T cells (NFAT) is an important factor, regulates many key T cell functions. In this study, we found that PML specifically enhanced the transcription activation of NFAT. In PML-null mouse embryonic fibroblasts, no transcription activity of NFAT could be detected. There was a selective requirement of PML isoform in NFAT activation. The additive effect of PML was not due to sequestration of Daxx by PML. PML specifically promoted the expression of many, but not all, NFAT targeted genes, including TNFa, TRAIL and Cbl-b. A specific binding of PML to NFATc was found by GST pull down assay and immuno-precipitation. The interaction of PML with NFATc in vivo was further confirmed by ChIP and DNA affinity precipitation assay (DAPA) analysis. Knockdown of PML was associated with reduced nuclear presence of NFAT. The unexpected coupling of PML with NFAT reveals a novel mechanism underlying the diverse physiological functions of PML.

2049/B514

Novel RNA Binding Properties of the MTG Chromatin Regulatory Proteins.

S. Rossetti[1], L. van Unen[2], N. Sacchi[1], A. T. Hoogeveen[2]; [1]Cancer Genetics Program, Roswell Park Cancer Institute, Buffalo, NY, [2]Clinical Genetics, Erasmus MC, Rotterdam, Netherlands

The myeloid translocation gene (MTG) proteins are non-DNA binding transcriptional regulators capable of interacting with chromatin modifying proteins. As a consequence of leukemia-associated chromosomal translocations, two of the MTG proteins, MTG8 and MTG16, are fused to the DNA-binding domain of AML1, a transcriptional activator crucial for hematopoiesis. The AML1-MTG fusion proteins, as the wild type MTGs, display four conserved homology regions (NHR1-4) related to the Drosophila nervy protein. Structural protein analysis led us to test the hypothesis that specific MTG domains may mediate RNA binding. By using an RNA-binding assay based on synthetic RNA homopolymers and a panel of MTG deletion mutants, here we show that all the MTG proteins can bind RNA. The RNA-binding properties can be traced to two regions: the Zinc finger domains in the NHR4, which mediate Zinc-dependent RNA-binding, and a novel short basic region (SBR) upstream of the NHR2, which mediates Zinc-independent RNA-binding. The two AML1-MTG fusion proteins, retaining both the Zinc fingers domains and the SBR, also display RNA-binding properties. Our findings open new perspectives on the possible involvement of an RNA component in MTG-mediated chromatin regulation. This work was supported through Erasmus MC funds (ATH), an AIRC grant (NS) and, in part, by the NCI Cancer Center Support Grant to Roswell Park Cancer Institute (CA016056). SR was partially supported by a postdoctoral fellowship from the University of Milan (Italy).

2050/B515

Yin Yang 1 (YY1) Activates BRCA1 Transcription Reflecting a Biological link between YY1 and BRCA1 Expression in Breast Cancer.

M. Lee, C. Deng; GDDB, NIH, Bethesda, MD

Breast cancer is a major cause of cancer mortality in women and affects approximately one in eight women during their lifetime. Approximately 90% of breast cancers are sporadic while the remaining 10% are inheritable. Importantly, the BRCA1 gene is mutationally inactivated in 50-90% of hereditary breast and ovarian cancers. Although BRCA1 mutations are rare in sporadic breast cancers, BRCA1 expression is reduced in many of these cancers. Sporadic breast cancers may exhibit decreased BRCA1 expression and display a 'mutant BRCA1-like' phenotype although there are no genetic deletions or changes in the methylation status of the BRCA1 locus. The molecular mechanisms leading to reduced BRCA1 expression and/or function in the sporadic breast cancers remain unclear. To investigate the cause of

reduced BRCA1 expression in sporadic breast cancers, we screened 876 transcription factors for their ability to regulate the BRCA1 promoter using a Genome-wide Full-length cDNA Array. Among several positive regulators the Gli-Krueppel-related transcription factor YY1 showed the most dramatic trans-activation of the BRCA1 promoter. By promoter deletion analysis we show that the -201/+39 bp region of the BRCA1 promoter mediates strong basal activity of the BRCA1 promoter in breast cancer cells and we identified a 40 bp element in the -201/-162 bp region that responds to YY1. The direct interaction of YY1 to this element was confirmed by gel mobility shift assays, site-directed mutagenesis, and chromatin immuno-precipitation analysis. Moreover, YY1 and BRCA1 proteins are expressed at similar levels and present in the nucleus of both normal mouse mammary cells and breast cancer cells. To assess clinical linkage between YY1 and BRCA1, we profiled the expression levels of YY1 and BRCA1 in 46 human breast cancers which are categorized as hyperplasias, in situ carcinomas and/or invasive cancers. Strikingly, there is a significant positive clinical correlation between the level of YY1 and BRCA1 expression in human breast tissues. Taken together, our findings suggest that YY1 is a key regulator of BRCA1 gene expression and may be causally linked to the molecular etiology of human breast cancer.

2051/B516

C-Terminal Binding Protein (Ctbp)-Mediated Transcriptional Repression Is Regulated by Inhibitor of Apoptosis Protein (IAP).

J. Lee, S. Yoo, Y. Kim, S. Lee; Department of Life and Nanopharmaceutical Sciences and Department of Biology, Kyung Hee University, Seoul, South Korea

Inhibitor of apoptosis proteins (IAPs) are known as the key negative regulators of apoptosis. They are evolutionarily conserved from Drosophila to human. They all have one-to-three Baculovirus IAP repeat (BIR) domain(s) responsible for binding to caspases and inhibiting their activity. Another domain in most IAPs is the Ring domain, which can function as an E3 ubiquitin ligase. To explore a novel function of IAPs, we sought to identify proteins that interact with Diap1 in Drosophila. In our study, we identified dCtBP (Drosophila C-terminal Binding Protein) which is a transcriptional co-repressor as a binding protein to Diap1. dCtBP was poly-ubiquitinated by Diap1 and degraded by proteasome dependent manner in S2 cell. We also found XIAP among five IAPs in human as an E3 ubiquitin ligase for human CtBP1 in HeLa. We investigated whether CtBP1-mediated transcriptional repression might be regulated by XIAP using luciferase reporter and RT-PCR of the genes repressed by CtBP1. Our results suggest that XIAP may play an important role in the regulation of CtBP1-mediated transcriptional repression by regulating CtBP1 protein level.

2052/B517

Repression of Fatty Acid Synthase by Hypoxia-Inducible Factor and Its Targets, DEC1 and DEC2.

S. Choi, H. Cho, H. Park; University of Seoul, Seoul, South Korea

Fatty acid synthase (FAS) is the key lipogenic enzyme responsible for the endogenous synthesis of fatty acids, which is regulated by the transcription factor, sterol regulatory element binding protein-1c (SREBP-1c). We demonstrated that Hypoxia-Inducible Factor (HIF) represses FAS by inducing Differentiated embryo chondrocyte 1 (DEC1) and DEC2, bHLH homodimeric transcription repressors. We found that both DEC1 and DEC2 prevent SREBP-1c from binding to the FAS promoter presumably by interacting with SREBP-1c protein. DEC2 is instantly and temporarily induced in acute hypoxia, while DEC1 is induced in prolonged hypoxia. This expression profile reflects the finding that DEC1 represses DEC2, thus maintains low level of DEC2 in prolonged hypoxia. DEC2-siRNA restores the hypoxic repression but DEC1-siRNA fails to do so, suggesting that DEC2 is the major initiator of hypoxic repression of FAS, whereas DEC1 substitutes for DEC2 in prolonged hypoxia. Our findings imply that DEC1 and DEC2 are the mediators to repress FAS gene in response to hypoxia. [Supported by a grant (R200706192003) from the Basic Research Program of the KOSEF]

2053/B518

Identification of a Physical Interaction between the Nuclear Envelope Protein Mps3p and the Histone Variant Htz1p in Saccharomyces Cerevisiae.

L. M. Antoniacci; Science, Marywood University, Scranton, PA

The nuclear envelope functions in several aspects of gene transcription and the spatial organization of chromatin. One component of the nuclear envelope, Mps3p, is directly involved in several of these functions including telomere clustering, telomere positioning, and telomere silencing via Mps3p interaction with the silencing protein Sir4p. Here we provide new evidence of an in vitro interaction between Mps3p and the histone variant protein Htz1p. Htz1p replaces the conventional H2A protein at genes that are clustered near telomeres. Htz1p has been documented as localizing to the promoters of inactive genes prior to their activation, and it has also been shown to prevent the spread of Sir proteins at telomere-proximal locations. The interaction between Mps3p and Htz1p may provide further insight into how proteins at the nuclear envelope may be playing a role in gene expression. The interaction suggests a role in telomere gene activation where Mps3p may help facilitate the removal of Sir proteins and addition of Htz1p to the telomere-proximal gene promoters at the nuclear envelope.

2054/B519

Analysis of the Lsm1 Mutants Provides Insight into The Mechanism Of Decapping.

A. Chowdhury, T. Sundaresan; Biochemistry & Molecular Biology, Uniformed Services University of Health Sciences, Rockville, MD

A major mRNA decay pathway conserved in all eukaryotes involves deadenylation followed by decapping that allows the 5' to 3' exonucleolytic degradation of the message body. Decapping is a key step in this 5' to 3' decay pathway. Here oligoadenylated but not polyadenylated mRNAs are selectively decapped in vivo and normal rates of decapping need the heteroheptameric Lsm1p-7p-Pat1p complex that has an intrinsic ability to recognize the oligo (A) tail. The molecular mechanisms in this decapping process are not known; presumably multiple sub steps are involved. In order to gain more insight into the mechanism of decapping, we have analyzed several decay defective yeast lsm1 mutants. In vitro RNA binding ability of the mutant complex is almost completely abolished in the case of lsm1-8 while in the case of lsm1-9 and lsm1-14, a moderate impairment of binding affinity was observed. By over expressing the corresponding lsm1 alleles, decay defect could not be suppressed in any of these mutants. Importantly, upon over expression in wild type background, lsm1-9 and lsm1-14 but not any other lsm1 allele including lsm1-8 allele could cause dominant inhibition of mRNA decay. These observations enable us to

distinguish at least two stages in this complex process namely binding of the Lsm1p-7p-Pat1 complex to the mRNA and post-binding events that ultimately lead to decapping.

2055/B520

A Novel Role for Iκbζ in the Regulation of Ifnγ Production.

R. M. Raices[1,2], Y. Kannan[1], S. Seshadri[1], V. Bellamkonda-Athmaram[1], M. D. Wewers[1]; [1]Integrated Biomedical Science Graduate Program, The Ohio State University, Columbus, OH, [2]Molecular Medicine, City of Hope, Duarte, CA

IκBζ is a novel member of the IκB family of NFκB regulators, which, unlike typical IκB proteins, modulates NFκB activity in the nucleus, rather than controlling its nuclear translocation. IκBζ expression has been shown to be specifically induced by IL-1R/TLR ligands and positively regulate NFκB-mediated transcription of genes such as IL-6 and NGAL. We recently reported that the caspase-1 dependent cytokines, IL-1β and IL-18, strongly synergize with TNFα for IFNγ production in the acute myelomonocytic KG-1 cell line, whereas the same cytokines alone have minimal effects on IFNγ production. Given the striking similarities between the IL-1R and IL-18R signaling pathways we hypothesized that a common gene product downstream of these receptors is responsible for the observed synergy between IL-1 cytokines (IL-1β and IL-18) and TNFα. We investigated IκBζ protein expression in KG-1 cells in response to IL-1β, IL-18 and TNFα stimulation and found that IL-1β and IL-18, but not TNFα, induced moderate IκBζ expression in KG-1 cells. However, when combined with IL-1β or IL-18, TNFα drastically synergized with these cytokines for IκBζ expression. These results correlate with our previous finding that IL-1β and IL-18 synergize with TNFα for IFNγ production. Finally, silencing of IκBζ expression lead to specific decreased IFNγ production. Overall, our data indicates the novel finding that IκBζ positively regulates IFNγ production in KG-1 cells in response to IL-1 cytokines and TNFα combined stimuli and may have great implications in understanding the signaling mechanisms that regulate cytokine production.

2056/B521

Models of Successive Levels of Resolution during Individual Gene Transcription.

J. Frenster, J. Hovsepian; Activator RNA Research, Physicians' Educational Series, Atherton, CA

Network simulation studies require integration of gene activity at several levels. Recent and on-going studies have revealed three levels of resolution showing pervasive interleaved transcription (Gingeras TR, "Mapping the strand-specific transcriptome of fission yeast", Nature Genetics 40: 935 (August, 2008). These studies have included the levels of multiple gene clusters, of overlapping primary transcripts, and of individual gene loci (ibid.). Newer studies have suggested a deeper level of resolution distinguishing transcription on one DNA strand from that on the opposing DNA strand within a single gene locus (Dutrow N, et al., "Dynamic transcriptome of S. pombe shown by RNA-DNA hybrid mapping", Nature Genetics 40: 977 (August, 2008). The products of such transcription include both coding and non-coding RNAs, sense and antisense orientations, and single and paired duplexes. Among the latter, the increasing importance of RNA-DNA and RNA-RNA duplexes during regulation of gene transcription is being recognized as crucial to gene selectivity and gene specificity. The recent studies of RNA-RNA interactions within promoter sites and enhancer sites of the same gene product exemplifies such research (Schwartz JC, et al., "Antisense transcripts are targets for activating small RNAs", Nature Structural & Molecular Biology, 15: 842 (August, 2008), as does the study of individual genes on one chromosome and throughout the euchromatin of one cell (Mikkelsen TJ, et al., "Dissecting direct reprogramming through integrative genomic analysis", Nature 454: 49 (July 3. 2008). A preliminary network simulation of these data for several linked genes reveals driving forces from initiation levels which determine kinetics thoughout the particular gene network. These diverse data re-assert the need and importance of high-resolution isolation techniques and comprehensive composite structures in analyzing network interactions within intact living cells.

RNA and RNA Localiztion (2057 – 2075)

2057/B522

Ultrastructural Visualization of Oskar mRNA Particle Assembly and Transport.

A. Trucco, A. Ephrussi; Developmental Biology, European Molecular Biology Laboratory, Heidelberg, Germany

Localization of *oskar* mRNA and its local translation at the posterior pole of the *Drosphila* oocyte are essential for development of the abdomen and germline of the fly. Despite a tremendous advance in genetic and biochemical identification of components involved in *oskar* mRNA localization, little is known about the cellular structures and mechanisms that mediate transport and anchoring of the mRNA to the posterior pole. We have carried out a detailed analysis at the ulstrastructural level of the subcellular distribution of the mRNA and associated proteins during oogenesis. We have determined that the endogenous *oskar* RNA is transported as “naked” RNP particles (RNPs) that undergo coalescence while moving from the anterior to the center and from the center to the posterior pole of the oocyte. Barentsz, Hrp48, and Staufen proteins, which are required for *oskar* localization, colocalize with *oskar* RNA. The *oskar* RNPs are also associated with microtubules (MTs) throughout the oocyte and colocalize with both Kinesin and Dynein. To gain further insight into the mechanisms regulating *oskar* transport, we studied *oskar* localization in different mutant backgrounds. In *kinesin heavy chain* mutants, the majority of *oskar* RNA accumulates in small particles along the lateral cortex. Strikingly, these RNPs retain their association with MTs and Dynein, suggesting that in the absence of Kinesin, Dynein drives the RNPs to the lateral cortex, where the MT have their minus ends. In *TropomyosinII* oocytes *oskar* also fails to reach the posterior pole and accumulates at the lateral cortex. Remarkably, *oskar* particles retain their association with MTs and Dynein, but no longer colocalize with Kinesin, indicating that cytoplasmic Tropomyosin, and possibly actin, has a role in stabilizing and/or recruiting Kinesin to *oskar* RNPs. Finally, in *barentsz* and *hrp48* mutants *oskar* is present in small RNPs dispersed throughout the oocyte cytoplasm. These particles are not associated with MTs and do not colocalize with Kinesin or Dynein. Thus, Barentsz and Hrp48 play a pivotal role in the assembly *oskar* RNPs competent for association with the motor proteins and MTs, and thus for transport to the posterior pole.

2058/B523

Export of Single B-Actin Mrnas in Living Cells.

D. Gruenwald[1,2], R. Singer[2,1]; [1]Gruss-Lipper Biophotonics Center, Albert Einstein College of Medicine, Bronx, NY, [2]ASB, Albert Einstein College of Medicine, Bronx, NY

All protein and RNP traffic between nucleus and cytoplasm occurs via nuclear pore complexes (NPC), extremely large (~120 MDa) nano-machines with a barrel-shaped geometry of 125 nm in diameter and a length of 120 nm. Transport cargo with weights greater than 40 kDa are translocated by the NPC in a signal-dependent manner. Using the MS2 system to label nascent mRNA transcripts we follow single mRNAs inside the nucleus and their export into the cytoplasm. Pom121-tdTomato is used to localize NPCs. mRNA and Nuclear Pores are imaged with a time resolution of 20 ms and a localization precision of 30 nm.

2059/B524

Host Nxf1 Is Crucial for Nuclear Export of Select Influenza mRNAs.

S. Bui, H. Medina R., L. L. Newcomb; Department of Biology, California State University San Bernardino, San Bernardino, CA

Influenza is a negative strand RNA virus which transcribes its mRNA in the nucleus and requires the splicing of two viral mRNAs and the nuclear export of three distinct classes of mRNAs; spliced, intron containing, and intron-less. In eukaryotes, cellular transcription is coupled to mRNA processing of the 5'cap structure, poly A tail, splicing and nuclear export (Strasser K.,2002). Influenza transcription on the other hand is carried out by the viral RNA dependent RNA polymerase, including generation of the 5' cap and poly A tail (Krug, R.M.,1989). Thus a major question regarding influenza biology persists; how are influenza mRNAs delivered to the host nuclear mRNA processing machinery for splicing and/or export? The study of viral mRNA export has identified two host cellular proteins, Nxf1 and Crm1, which represent two separate nuclear export pathways (Pasquinelli A.E.,1997 and Neville M.,1997). Evidence also supports existence of alternate nuclear export pathways (Paca R.E.,2000). Influenza mRNA export is not obstructed by Crm1 inhibitors (Elton D., 2001); therefore we set out to examine the role of host Nxf1. We first established the time course of specific influenza mRNA export to the cytoplasm via RT-PCR. To discover if export of any viral early mRNAs utilize the Nxf1 pathway, we expressed dominant negative Nxf1 protein (Kang Y, 1999) in A549 cells prior to infection with influenza A Udorn and collected cytoplasmic RNA at 3 hours post infection. RT-PCR shows that influenza NP mRNA is decreased in the cytoplasm while PA and PB1 mRNA are not. In order to test direct NP mRNA association with Nxf1 protein during viral infection, we expressed a Flag-tagged Nxf1 protein (Huang Y, 2004) in A549 cells prior to infection with influenza A Udorn, and immunoprecipitated Nxf1/RNA complexes using anti-Flag antibody. We find influenza NP mRNA is specifically co-immunoprecipitated with Nxf1. Taken together, our results reveal that influenza intron-less NP mRNA exports via host Nxf1 while other early expressed intron-less mRNAs, PA and PB1, do not, suggesting these viral mRNAs must utilize an alternate nuclear export pathway. We are the first to report on the crucial role of host Nxf1 in influenza NP mRNA export.

2060/B525

Hepatitis E Virus (HEV) Biology: Time Kinetics of Replication.

S. K. Panda, H. Durgapal, P. K. Varma, A. Kumar, N. Kapur; Pathology, All India Institute of Medical Sciences, New Delhi, India

Hepatitis E Virus (HEV) is a positive strand RNA virus, responsible for viral hepatitis in humans. Only a hypothetical model is available for the replication and expression cycle of HEV. We investigated the replication time kinetics of the virus using a replicon of HEV developed by us. Positive strand RNA viruses replicate through a negative sense RNA intermediate. We used confocal time lapse microscopy on live HepG2 cells transfected with capped polyadenylated HEV replicon along with molecular beacon (BHQ2-6064-6083-Alexa546, designed by us) specific for the negative sense replicative intermediate. In another set of experiments we quantified positive sense genomic and sub genomic RNA every two hours post transfection by qRT-PCR (Quantitative Real Time PCR) using primers starting at positions 2589, 4554, and 6880. In our live cell imaging experiment we found maximum intensity of signals between 6 to 7 hours post transfection showing accumulation of negative sense RNA. The qRT-PCR data showed positive sense RNA increasing from 8 hours post transfection. These results show a time lag between synthesis of negative and positive sense viral RNA. The qRT-PCR data also demonstrated presence of only one sub genomic RNA and a higher fold increase of sub genomic RNA over genomic RNA. We were able to analyse the time kinetics of HEV replicon both with live cell confocal imaging and qRT-PCR. This is possibly the first time anybody has detected specific RNA as it is being synthesized. The method not only has implication on studies of virus biology but can impact studies on developmental biology as well.

2061/B526

Identification of Hnrnp-Q1 as a Novel β-Actin mRNA Binding Protein within RNA Transport Granules.

L. Xing, X. Yao, A. Swanson, Y. Sasaki, G. Bassell; Departments of Cell Biology and Neurology, Emory University School of Medicine, Atlanta, GA

The active transport and local translation of β-actin RNA provide mechanisms to promote the enrichment of β-actin protein in neuronal growth cones and filopodia, which has been shown to be important for regulation of axon guidance and synapse formation. Previous studies have characterized a molecular interaction between zipcode binding protein-1 (ZBP1) and the β-actin mRNA zipcode, within the 3'UTR, which is necessary for the transport and translational regulation of β-actin mRNA. However whether trans-acting factors other than ZBP1 may also be important for the transport or translational control of β-actin mRNA in neuronal processes is unclear. In this study, we identified hnRNP-Q1 (also known as Syncrip or Nsap1) as a novel β-actin mRNA interacting protein. hnRNP-Q1 and hnRNP-Q3 are derived by alternative splicing. Subcellular fractionation analysis showed that hnRNP-Q1 was mainly localized in the cytoplasm whereas hnRNP-Q3 was highly enriched in the nucleus. Using immunofluorescence staining with hnRNP-Q1 specific antibodies, we found that hnRNP-Q1 granules were localized in both axons and dendrites and associated with microtubules in processes and growth cones of cultured hippocampal neurons. In live neurons, overexpressed EGFP-tagged hnRNP-Q1 granules exhibited fast, bidirectional movements in neuronal processes. Results from our biochemical studies showed that hnRNP-Q1 associated with ZBP1 and β-actin mRNA in vivo and the interaction of hnRNP-Q1 and β-actin mRNA was mediated through a 3'UTR sequence that is distinct from the β-actin mRNA zipcode. These observations

suggest that hnRNP-Q1, possibly together with ZBP1, form mRNP granules with β-actin mRNA which may be required for efficient transport or precise translational regulation of β-actin mRNA in response to external stimuli. Supported by NIH RO1 HD046368 and Spinal Muscular Atrophy Foundation grants to GJB.

2062/B527

Analyzing the Function of Microtubule Localized RNAs during Mitosis.

M. D. Blower; [1]Molecular Biology, MGH, Boston, MA, [2]Department of Genetics, Harvard Medical School, Boston, MA

RNA serves a wide variety of functions within cells, acting both as genetic information, in the form of mRNA, and as a structural and catalytic component of various protein complexes. Posttranscriptional regulation of both mRNAs and noncoding RNAs through transport to specific subcellular locations is a widely used mechanism for modulating gene expression in time and space. Work from our lab has demonstrated that a specific subset of mRNAs are localized to microtubules during mitosis in vertebrates (MT-RNAs). MT-RNAs are enriched for mRNAs involved in regulation of mitotic progression and various aspects of developmental patterning. To determine if the mitotic spindle serves to partition specific mRNAs symmetrically or asymmetrically during early cell divisions we have injected fluorescently labeled mRNAs into early Xenopus embryos and followed their localization during early development. Our preliminary results suggest that some MT-RNAs are localized asymmetrically while others are uniformly distributed during development. Experiments are currently underway to determine if microtubule localization of mRNAs is important for embryonic patterning. To understand how specific mRNAs are localized to mitotic microtubules we have begun to map cis-acting localization elements located in MT-RNAs. Surprisingly, we have found that the localization element of several mRNAs is located within the protein-coding portion of the mRNA, but that mRNA localization is translation independent, while other localization elements are located in the 3'UTR of the mRNA. These results suggest that multiple different localization pathways contribute to localization of mRNAs to mitotic microtubules. Our work has shown that specific mRNAs are localized to mitotic microtubules, but it is possible that other classes of RNA (rRNA, tRNA, miRNA) are also enriched on microtubules and could contribute to the role of RNA in spindle assembly. We are currently employing large-scale sequencing technologies to identify all types of RNA that are localized to mitotic microtubules. We will present preliminary data describing spindle localization of noncoding RNAs.

2063/B528

Microtubule-Dependent Association of AKAP350A and CCAR1 with RNA Stress Granules.

E. Kolobova[1], A. Efimov[1], I. Kaverina[1], A. K. Rishi[4], J. Schrader[3], A. Ham[1], M. Larocca[2], J. Goldenring[1]; [1]Vanderbilt, Nashville, TN, [2]Universidad Nacional de Rosario, Rosario, Argentina, [3]University of British Columbia, Vancouver, BC, Canada, [4]Karmanos Cancer Institute, Detroit, MI

Recent investigations have highlighted the importance of subcellular localization of mRNAs to cell function. While AKAP350A, a multifunctional scaffolding protein, localizes to the Golgi apparatus and centrosomes, we have now identified a cytosolic pool of AKAP350A. Analysis of AKAP350A scaffolded complexes revealed two novel interacting proteins, CCAR1 and caprin-1. CCAR1, caprin-1 and AKAP350A along with G3BP, a stress granule marker, relocate to RNA stress granules after arsenite treatment. Stress also caused loss of AKAP350 from the Golgi and fragmentation of the Golgi apparatus. Disruption of microtubules with nocodazole altered stress granule formation and changes their morphology by preventing fusion of stress granules. In the presence of nocodazole, arsenite induces smaller granules with the vast majority of AKAP350A and CCAR1 separated from G3BP-containing granules. Similar to nocodazole treatment, reduction of AKAP350A expression also altered the size and number of G3BP-containing stress granules induced by arsenite treatment. A limited set of 69 mRNA transcripts was immunisolated with AKAP350A even in the absence of stress, suggesting the association of AKAP350A with mRNA transcripts. These results provide the first evidence for the microtubule dependent association of AKAP350A and CCAR1 with RNA stress granules.

2064/B529

SCYL1 Facilitates Nuclear tRNA Export in Mammalian Cells.

S. Chafe, D. Mangroo; University of Guelph, Guelph, ON, Canada

SCYL1 is a mammalian protein that is structurally homologous to an uncharacterized ORF found in all eukaryotic genomes sequenced to date. These proteins have a characteristic protein kinase-like domain at the N-terminus and a middle HEAT repeat domain, which is commonly involved in protein-protein interaction. However, SCYL1 and the orthologues are not protein kinases. Recent studies have shown that SCYL1 plays an important role in normal neuronal development, and COP1-meditated retrograde protein trafficking. Furthermore, the *S. cerevisiae* orthologue Cex1p has been shown to be a cytoplasmic component of the nuclear tRNA export process. We therefore investigated whether the human SCYL1 is required for nuclear tRNA export. Like Cex1p, overexpression of SCYL1 facilitated nuclear export of a nuclear export-defective mutant tRNA in *S. cerevisiae*. In mammalian cells, SCYL1 rescued the nuclear export defect of a human serine amber suppressor tRNA. Furthermore, SCYL1 was found to bind to tRNA directly and saturably *in vitro*. SCYL1 is located primarily in the cytoplasm, but a small amount of the protein was detected at the nuclear pore complex (NPC) by co-localization immunofluorescence analyses. Co-immunoprecipitation experiments using HeLa cell extracts revealed that SCYL1 co-purifies with the primary nuclear tRNA export receptor, Exportin-t, along with Exportin-5, Ran, EF-1A, and Nup98. *In vitro* protein binding studies indicated that SCYL1 interacts directly with Exportin-t, the RanGTPase, as well as the nucleoporin, Nup98. Furthermore, SCYL1 was found to be part of a quaternary complex reconstituted *in vitro* consisting of Exportin-t, RanGTP, and tRNA, suggesting that SCYL1 is playing a role in the cytoplasmic phase of the nuclear tRNA export pathway. By analogy to Cex1p, SCYL1 may be involved in unloading aminoacyl-tRNAs from the export receptor at the cytoplasmic side of the NPC, and delivering it to EF-1A with the assistance of an unidentified intermediate.

2065/B530

Retrograde Movement of tRNA from the Cytoplasm to the Nucleus May Not Be Universally Conserved.

A. D. Johnstone, S. Chafe, J. B. Pierce, R. T. Mullen, D. Mangroo; Molecular and Cellular Biology, University of Guelph, Guelph, ON, Canada

The intracellular trafficking of tRNA has long been thought to be a one-way trip from the site of biogenesis in the nucleus to the translation machinery in the cytoplasm. This view has recently been challenged by the findings that retrograded tRNA accumulates in the nuclei of *Saccharomyces cerevisiae* and a rat hepatoma cell line (Shaheen et al., 2007; Hurto et al., 2007; Whitney et al., 2007; Shaheen and Hopper, 2005). Contrary to these findings, we have obtained data suggesting the tRNA retrograde process does not occur in several plant and mammalian cell lines, including rat hepatoma cells. This apparent absence of retrograde tRNA movement from the cytoplasm to the nucleus in plants and mammals may be related to the nuclear localization of the tRNA splicing machinery in these cells, which unlike *S. cerevisiae* do not require nuclear export and subsequent re-import of tRNAs during the maturation process. Overall, these findings call into question the universality of retrograde tRNA movement and suggest this phenomenon may be limited to *S. cerevisiae*.

2066/B531

A Nucleostemin-Like Gtpase in Drosophila Melanogaster.

R. Rosby[1], P. DiMario[1], V. L. Robinson[2]; [1]Biological Sciences, Louisiana State University, Baton Rouge, LA, [2]Molecular and Cell Biology, University of Connecticut, Storrs, CT

Mammalian Nucleostemin (NS) is a nucleolar GTP-binding protein expressed in various stem cells and cancer cells. NS declines in abundance as progenitor cells differentiate, and it interacts with p53 to regulate cell cycle progression. We describe a Nucleostemin-like protein (NS1) in Drosophila melanogaster encoded by conceptual gene, CG3983. NS1 is 33% identical and 67% similar to human NS; highest conservations were found in the amino terminal basic domain and in the circularly permuted GTP-binding domains. We show that Drosophila NS1 has intrinsic GTPase and ATPase activities. Immuno-fluorescence microscopy showed robust NS1 expression in larval midgut island cells that proliferate during pupation to form the adult midgut, and in larval brain cells and imaginal (progenitor) disc cells. Other larval and adult cell types showed weaker NS1 expression. Over-expressed GFP-NS1 localized to the granular regions of nucleoli, the nucleoplasm, and to banded regions of polytene chromosomes. RNAi knockdown of NS1 caused nucleolar accumulation of GFP-RpL11 and mRFP-RpL26. The observation suggests that NS1 functions in the nucleolus-to-nucleoplasm transfer of the large ribosomal subunit. The formation of refractive, neutral red-positive vesicles in cells lacking NS1 suggested an autophagic response. Autophagy induction has been well characterized with the loss of mTOR, which suggests that depletion of NS1/ribosome function inhibits mTOR thus activating autophagy.

2067/B532

A Role for Polypyrimidine Tract Binding Protein in the Establishment of Focal Adhesions.

I. Babic, S. Sharma, D. Black; Dept. of MIMG, UCLA/HHMI, Los Angeles, CA

Polypyrimidine tract binding protein (PTB) is an RNA binding protein with a well-characterized role as a repressor of splicing of alternative exons. However, PTB is a multifunctional protein with several reported cytoplasmic functions. Within the cytoplasm, PTB has been reported to regulate stability and translation of specific mRNAs, and to have a role in transport and localization of Vg1 mRNA to the vegetal pole of Xenopus oocytes. PTB has also been identified as one of several RNA binding proteins associating with the focal adhesion protein paxillin during the early stages of cell spreading (1). Upon adhesion of mouse embryo fibroblasts (MEFs) to fibronectin, we observe a transient re-localization of nuclear PTB to the cytoplasm. We also observe PTB in newly formed cell protrusions. PTB re-localizes back to the nucleus as the cells become well spread with fully developed focal adhesions. Reducing PTB levels in these MEFs leads to a decrease in vinculin staining at the cell periphery and decreased number of cell protrusions. By UV crosslinking and immunoprecipitation we isolated PTB associated transcripts from the cytoplasm of cells undergoing adhesion and early spreading. Interestingly, transcripts encoding the focal adhesion scaffolding proteins vinculin, talin and alpha-actinin were identified in PTB immunoprecipitates. These transcripts contain conserved polypyrimidine tracts within their 3'UTRs that we demonstrate can directly bind PTB. Knockdown of PTB does not affect vinculin mRNA or protein levels, but limits the size and number of vinculin containing cell protrusions. By immunofluorescence combined with fluorescence in situ hybridization (FISH) we show that vinculin mRNA and PTB protein co-localize in the cytoplasm of adhering cells and at sites of cell protrusion during early cell spreading. We propose a model whereby PTB plays a role in localizing mRNAs encoding focal adhesion scaffolding proteins to sites of cell protrusion formation during the initial stages of cell spreading. Loss of PTB may mislocalize these transcripts leading to limited focal adhesion assembly and reduced formation of cell protrusions. (1) de Hoog, Foster, and Mann. 2004. Cell 117, 649-662.

2068/B533

Distinct Roles for Khd1p, the Yeast Ortholog of Mammalian Hnrnp K, in the Localization and Expression of Bud-localized mRNAs in yeast.

K. Irie[1], Y. Hasegawa[1], A. P. Gerber[2]; [1]Department of Molecular Cell Biology, University of Tsukuba, Tsukuba, Japan, [2]Institute of Pharmaceutical Sciences, ETH Zurich, Zurich, Switzerland

RNA localization is a widespread mechanism to achieve localized protein synthesis. In yeast Saccharomyces cerevisiae, Ash1p is a specific repressor of transcription that localizes asymmetrically to the daughter cell nucleus through the active transport of ASH1 mRNA to the bud tip. This localization, as well as that of 21 other mRNA, depends on the actin cytoskeleton and five She proteins. The RNA-binding protein Khd1p (KH-domain protein 1), the yeast ortholog of mammalian hnRNP K, is required for efficient localization of ASH1 mRNA to the bud-tip probably acting as a translational repressor during mRNA transport. We have systematically examined Khd1p mRNA targets and co-localization with known bud-tip localized mRNAs in vivo. Affinity purification and DNA microarray analysis of Khd1p-associated mRNAs revealed hundreds of potential mRNAs targets, many of them encoding membrane-associated proteins. The putative targets include the messages for MID2, MTL1, WSC2, SRL1, EGT2, CLB2, ASH1, and Khd1p co-localizes with these mRNAs at the bud-tip. The combination of bioinformatics, RNA localization and in vitro RNA-binding assays revealed that Khd1p binds to CNN repeats in coding regions of mRNA targets. Among the proteins encoded by previously known bud-tip localized mRNAs, only Mtl1p levels were decreased in khd1Δ mutant

cells whereas Ash1p and Srl1p were reduced in cells overexpressing KHD1. Hence, Khd1p differentially affects gene expression possibly due to combinatorial arrangement with additional factors reflecting the redundant structure of post-transcriptional regulatory systems.

2069/B534

Core Exosome-Independent Roles for dRrp6 in Mitotic Progression.

A. C. Graham, D. L. Kiss, E. D. Andrulis; Molecular Biology & Microbiology, Case Western Reserve University, Cleveland, OH

Exosome complexes are 3' to 5' exoribonucleases composed of subunits that are critical for numerous and distinct RNA metabolic pathways. Several studies have implicated exosome subunits in chromosome segregation and cell division but the functional relevance of these findings remains unclear. Here, we report that, in *Drosophila* melanogaster S2 tissue culture cells, dRrp6 is required for cell proliferation and error-free mitosis, but the core exosome subunit dRrp40 is not. Micorarray analysis of dRrp6-depleted cells (by RNAi) reveals increased levels of cell cycle- and mitosis-related transcripts. Also, depletion of dRrp6 elicits a decrease in the frequency of mitotic cells and in the mitotic marker phospho-histone H3 (pH3) protein levels. Concomitantly, an increase in chromosome congression, separation, and segregation defects are observed. Endogenous dRrp6 dynamically redistributes during mitosis accumulating predominantly, but not exclusively, on the condensed chromosomes. In contrast, most core subunits localize predominantly to microtubules (MTs) throughout cell division. Finally, dRrp6-depleted cells treated with MT poisons exhibit normal kinetochore recruitment of the spindle assembly checkpoint protein, BubR1, without restoring pH3 levels. This suggests that these cells undergo premature chromosome condensation. Collectively, these data support the idea that dRrp6 has a core exosome-independent role in cell cycle and mitotic progression.

2070/B535

Gemin3 Is an Essential Gene Required for Larval Motor Function and Pupation in Drosophila.

K. Shpargel[3,2], K. Praveen[2], T. Rajendra[1], A. Matera[1,2]; [1]Biology, Univ of North Carolina, Chapel Hill, NC, [2]Genetics, Univ of North Carolina, Chapel Hill, NC, [3]Genetics, Case Western Reserve Univ, Cleveland, OH

The assembly of metazoan Sm-class small nuclear ribonucleoproteins (snRNPs) is an elaborate, step-wise process that takes place in multiple subcellular compartments. The initial steps, including formation of the core RNP, are mediated by the Survival Motor Neuron (SMN) protein complex. Loss-of-function mutations in human *SMN1* result in a neuromuscular disease called Spinal Muscular Atrophy. The SMN complex is comprised of SMN and a number of tightly-associated proteins, collectively called Gemins. In this report, we identify and characterize the fruitfly ortholog of the DEAD box protein, Gemin3. *Drosophila* Gemin3 (dGemin3) colocalizes and physically interacts with dSMN in vitro and in vivo. Furthermore, depletion of dGemin3 by RNA interference co-depletes dSMN and inhibits efficient Sm core assembly in vitro. Transposon insertion mutations in *Gemin3* also co-deplete dSMN and are lethal during the second instar larval stages. Transgenic overexpression of dGemin3 rescues the lethality, but overexpression of dSMN does not. The *Gemin3* mutants exhibit significant larval motor defects. Remarkably, appreciable numbers of the *Gemin3* mutants (along with one previously undescribed *Smn* allele) survive as larvae for several weeks without undergoing pupation. Our results demonstrate the conservation of Gemin3 protein function in metazoan snRNP assembly and reveal that loss of either *Smn* or *Gemin3* can contribute to neuromuscular dysfunction.

2071/B536

The RNA Binding Activity of the Multifunctional Protein, p54nrb, Is Modulated by Phosphorylation in a Cell Cycle-dependent Manner.

C. Bruelle, M. Bédard, M. Vincent; Dpt Medecine and CREFSIP, Université laval, Québec, QC, Canada

The DNA/RNA-binding protein p54nrb is known to participate in several nuclear processes such as transcriptional control, RNA splicing and editing, and DNA unwinding and pairing. Transcription and RNA maturation are closely coupled processes and both p54nrb and its partner, PSF, bind the carboxy-terminal domain of RNA polymerase II. Our data have shown that p54nrb is multi phosphorylated during mitosis, and a substrate of the mitotic regulator Pin1. Since transcription and splicing are inhibited during mitosis, we hypothesize that p54nrb is involved in this repression by regulation of its phosphorylation level. Our main objective is to determine the functions of p54nrb phosphorylation vis-à-vis mitotic repression in connection with our finding that a CDK1-mediated interaction between Pin1 and p54nrb is specific to mitotically arrested cells. Localization, protein interaction and RNA binding properties were studied in vitro and in vivo using phosphorylation mutants of p54nrb in interphase and mitotic conditions. No changes of p54nrb localization and protein interaction were observed in experiments using phosphorylation mutants of p54nrb. Conversely, nucleic acid binding studies showed that p54nrb phosphorylated in vitro by CDK1 had less affinity for homoribopolymeres. Cell fractionation experiments were performed using interphase or mitotic cells. Whereas interphase p54nrb resisted detergent extraction and solubilized only after RNase digestion, the majority of mitotic p54nrb fractionated in the detergent fraction. The RNA binding activity of p54nrb is regulated by phosphorylation with CDK1. This mechanism could contribute to the transcriptional repression observed in mitosis. As p54nrb and the prolyl isomerase Pin1 associate during mitosis, their potential role as general mitotic repressors of gene expression warrants further study.

2072/B537

Analysis of Yeast Chimeric Rrnas Harboring Drosophila D7a Expansion Segment Substitutions Within the Binding Site for Ribosomal Protein L25.

V. Ware, M. Kearse; Biological Sciences, Lehigh University, Bethlehem, PA

Although a conserved rRNA core structure and ribosomal protein families are the hallmark of the ribosome, it is clear that prominent structural differences within rRNA and ribosomal protein components exist between lineages that may contribute to interspecific differences in ribosome assembly, ribosome function, or the regulation of protein synthesis. Our interests are focused on determining how structural variation in L25/L23a ribosomal protein family and its 28S rRNA binding site impacts ribosome assembly and function. The L23a binding site is bisected by the presence of the highly divergent D7a rRNA expansion segment, which in some lineages (including *Drosophila*) is subject to a maturation event called "gap processing", creating 28Sα and 28Sβ rRNAs. Within some insect lineages, there has been mutual expansion of both RpL23a and the D7a segment, suggesting co-evolution of these components. The factors that limit D7a size expansion

within a given lineage are unknown; however, it is presumed that a D7a expansion threshold must exist above which rRNA-protein binding and/or ribosome assembly and function would be disrupted. As a test of the structural limits of the D7a expansion segment within yeast rRNA, we have designed a series of chimeric yeast rDNA constructs (using a high copy number plasmid carrying a GAL7-35S rRNA gene) for expression within different yeast strains to determine the impact of D7a structural complexity on ribosome biogenesis and function. In each construct the yeast D7a region was replaced with *Drosophila* D7a sequences, effectively introducing a fly-specific "tag". Following galactose induction of transfected wildtype yeast strains, chimeric yeast/fly construct expression was determined by RT-PCR. Preliminary results show differences in chimeric rDNA expression, likely due to differences in rRNA stability. D7a structure may have an effect rRNA interactions with yeast L25, leading to rRNA degradation. Expression of constructs within a yeast L25 chromosomal knock-out strain that is dependent on fly L23a for ribosome function and viability will determine if the presence of fly L23a alters chimeric rRNA maturation and stability. *Supported by research funds from Lehigh University*.

2073/B538

Proteomic Analysis of Nab2-Containing mRNP Complexes from S. Cerevisiae.

S. Carmody[1], M. Oeffinger[2], K. Wei[2], M. Rout[2], S. Wente[1]; [1]Cell and Developental Biology, Vanderbilt University, Nashville, TN, [2]Laboratory of Cellular and Structural Biology, The Rockefeller University, New York, NY

Nuclear export of mRNA is a highly regulated and dynamic process, whereby only properly matured transcripts are targeted for efficient transport into the cytoplasm. Many protein factors are known to be necessary for proper execution, including the essential family of heterogeneous nuclear ribonucleoproteins (hnRNPs). In vertebrates there are ~30 hnRNPs, and in yeast, there are ~10. Viewed as a stepwise mechanism: (i) pre-mRNA associates with hnRNPs co-transcriptionally, (ii) additional RNA-binding proteins are recruited during splicing and processing steps, (iii) the mature, export competent, mRNA-hnRNP (mRNP) complex is targeted to the NPC, and (iv) the mRNP is moved through the nuclear pore complex for cytoplasmic release. The hnRNP composition of the mRNP is predicted at each step. However, the exact biochemical nature of an export competent mRNP has not been defined. To address this question, we have employed a proteomics based approach in the budding yeast S. cerevisiae to target mRNPs containing an epitope tagged version of Nab2. Nab2 is a shuttling mRNA binding protein, whose export is dependent on RNA polymerase II transcription, and is essential for both viability and mRNA export. Using a novel lysis and purification strategy, Nab2-containing mRNPs were isolated from wild type cells, as well as from mutants blocked at distinct steps in mRNA export. The protein composition of the isolated Nab2-complexes was determined by a panel of mass spectrometry approaches. Validating our experimental approach, a number of mRNA binding proteins co-purified with Nab2, as well as a many components of the nuclear pore complex. Moreover, on a global level, the set of co-purifying proteins varied between wild-type cells and those deficient for mRNA export. Quantitative comparison pinpointed the proteomic differences in the Nab2-co-purifying proteins from distinct mutants. We speculate that these differences represent discrete protein-protein and/or protein-RNA interaction dynamics during the mRNA export mechanism.

2074/B539

Non-Coding ncRNA-Driven Episcription and Hermeneutics of the Genome: Deciphering Epigenetic [Non-Mendelian] Codes for Tissue Vascularization and Tolerance Entangled in the Cancer-Angiogenesis Epigenome.

J. H. Wissler; ARCONS Institute for Applied Research & Didactics, Postfach 1327, D-61231 Bad Nauheim, jhw@arcons-research.de, Germany

OBJECTIVE: Structural codes *[3D-episcripts]* for epigenetic phenotyping which escaped deciphering, so far: The genome is an orchestrated 3D-nucleic acid-[glyco-]protein nanomachine. It is not restricted to transcription *and* translation of 1D-DNA scripts [Mendelian code]. By episcription, the same genotype [e.g. twins] delivers a plethora of not identical epigenetic [non-Mendelian] phenotype variations. Functions of *endogenous* RNA isolated from total transcriptional output upon cell activation were investigated in entitled phenomena. ***METHODS:*** Wissler et al, Protides Biol.Fluids **34**:517-536,1986; Materialwiss.Werkstofftech.**32**:984-1008,2001; Ann.N.Y.Acad.Sci.**961**:292-297,2002; **1022**:163-184,2004; **1137**:2008. ***RESULTS: Functional small ncRNA*** [<200n] and RNP were found upon cell activation by extrinsic environmental factors, including mitogenic, cell-mediated immune memory, metabolic [hypoxia] and [physical] shear stress reactions. They comprise conventional models for epigenetic vascular remodeling variations directed rather to proteinaceous gene expression and regulation than genomic DNA sequence changes. Some edited, modified, *redox- and metalloregulated small hairpin* nc-shRNA bioaptamers are ***not complimentary*** to known genomic DNA sequences, but feature 3D-episcript codes to fit or misfit to distinct protein conformer phases. Some address homologous helix-nucleating structural [proteomic] domains, termed **K**/**R**xxx**H** [**K**/**R**3**H**], i.e.-t/s/x**K**/**R**/q/n/hxxx**H**/y/n/q/e/d/r/kx$_{7-9}$h/xx$_{7-9}$h/xx$_{5-20}$**K**/**R**/q/n/e/h- with accessory canonical basic [R/K]$_n$, R/K-zipper, SR/K/RS, EF-hand and/or HxxxH/y/n/q segments, shared in several epigenetic regulator proteins entangled in growth, metabolic syndromes, vascular, cancer epi- and genetic information indexing of the epigenome. ***CONCLUSIONS:*** The results suggest epigenetic [non-Mendelian] codes consisting of 2 different associated imprints: [1] Nucleic acid 3D-episcripts [e.g. ncRNA] as derivatives from but not directly retranslatable to Mendelian codes. [2] Defined domains in epigenetic regulator proteins of Mendelian origin as interaction address with [1]. This suggests a tolerated mess-chaotic tumor vascularization as *bioaptamer disorder* in ncRNA-switched proteinaceous genetic and epigenetic processes.

2075/B540

DNA Methylation and Cellular Response to Heat Shock.

S. M. Levesque, M. Paulin-Levasseur; Biology, University of Ottawa, Ottawa, ON, Canada

Human diseases such as Hutchinson-Gilford Progeria Syndrome (HPGS) are classified as laminopathies. With respect to this ailment, the root cause is described as a mutation within the lamin A gene giving rise to a truncated protein which accumulates in the nucleus causing premature aging. Through a mechanism still poorly understood, this will affect the epigenetic control of the cell. Epigenetic control such as DNA methylation relies on DNA methyltransferase (DNMT) enzymes which usually results in transcriptional silencing of genes. Exposure to environmental stress such as heat shock implies modifications on cell structure and function induced for survival. Human cells display a stress-defense mechanism through the formation of ribonucleoprotein aggregates known as nuclear stress bodies (nSBs). During nSB

assembly, heterochromatic silent satellite III sequences become actively transcribed into stable RNAs which associate with RNA-binding proteins including the serine-arginine rich (SR) protein SF2/ASF. The objective of this study is to determine if cells displaying distinct epigenetic status i.e.: varying states of DNA methylation, respond differently to environmental stresses. Five cell lines known to possess varying epigenetic status were examined by targeting SF2/ASF as a marker for nSB formation. Prior to heat shock, immunofluorescence microscopy revealed that SF2/ASF is uniformly distributed within the nucleus. Moreover, Western blot analysis reveals statistically significant differences in the expression of SF2/ASF between cell lines before heat shock. However, following a one hour heat shock at 42°C we detect different numbers of nSBs in all cell lines varying between 2 and 15 nSBs. In addition, a statistically significant reduction in SF2/ASF expression is noted. The reduction in SF2/ASF expression leads us to conclude that the particular epigenetic status of a cell will directly affect the manner in which it will respond to environmental stimuli such as heat shock. However changes if any in stress response are still not understood. Further inquiries in this matter are needed.

Structure of the Nuclear Envelope II (2076 – 2095)

2076/B541

Trans-Membrane Actin-Associated Nuclear Envelope (TAN) Lines of Nesprin-2 Giant and SUN2 Are Novel Structures Required for Rearward Nuclear Movement during Centrosome Orientation in Migrating Cells.

G. Luxton[1], E. R. Gomes[2], E. Folker[1], G. Gundersen[1]; [1]Pathology and Cell Biology, Columbia University, New York City, NY, [2]UMR S 787-Groupe Myologie, University Pierre et Marie Curie, Paris, France

At the onset of cell migration, retrograde actin flow moves the nucleus to the cell rear orienting the centrosome and Golgi apparatus between the leading edge and the nucleus. How actin flow is coupled to the nucleus to move it is unclear. We tested whether the LINC (linker of nucleoskeleton and cytoskeleton) complex, which consists of the inner nuclear membrane proteins, SUN1 and SUN2, and the actin-binding outer nuclear membrane protein, nesprin-2 giant (N2G), was involved in nuclear movement. SUN1, SUN2, and N2G were required for nuclear movement, centrosome orientation and efficient cell migration as determined through the use of dominant negative constructs and siRNA. Expression of a GFP-N2G construct (mini-N2G) encoding the KASH and the actin-binding calponin-homology (CH) domains restored nuclear movement in N2G depleted cells, whereas mini-N2G lacking functional CH domains did not. Surprisingly, mini-N2G assembled into linear arrays along actin cables localized on the dorsal and ventral surfaces of the nucleus. These linear mN2G assemblies were actin-dependent as cytochalasin-D and CH mutants inhibited their formation. FRAP studies showed that mini-N2G in these assemblies was immobilized relative to bulk nuclear envelope mini-N2G. Endogenous N2G and SUN2 were also detected in linear membrane assemblies that colocalized with actin cables on the nucleus. We refer to these novel assemblies as trans-membrane actin-associated nuclear envelope (TAN) lines. Live imaging of dorsal TAN lines (labeled with either mini-N2G or GFP-SUN2) showed that they move at the same rate as the nucleus and actin retrograde flow. SUN2 depletion did not affect mini-N2G TAN line formation or movement; however, the TAN lines appeared to slip over the nucleus while the nucleus remained immobile. These results show that the LINC complex forms a novel actin-associated structure on the nuclear membrane that is required for nuclear movement during centrosome orientation and suggest that specific positioning of the nucleus is important for cell migration.

2077/B542

Non-Farnesylated Prelamin a Mislocalization and Disorganization of Heterochromatin Marker Trimethyl-K9-Histone 3 in Emery-Dreifuss Muscular Dystrophy Fibroblasts after Prelamin a Interfering Drug Treatment.

C. Capanni[1], M. Columbaro[1], S. Squarzoni[1], D. Camozzi[2], E. Schena[2], M. Wehnert[3], N. Maraldi[2], G. Lattanzi[1]; [1]IGM Unit of Bologna c/o IOR, CNR, Bologna, Italy, [2]Laboratory of Cell Biology and Electron Microscopy, Istituto Ortopedico Rizzoli, Bologna, Italy, [3]Institute of Human Genetics, University of Greifswald, Greifswald, Germany

Emery-Dreifuss muscular dystrophy (EDMD) is a heterogeneous late-onset disease involving skeletal muscle wasting and heart defects caused by mutations of the inner nuclear membrane emerin (EDMD1), nuclear lamina components lamin A/C (EDMD2) or in the inner nuclear membrane binding proteins nesprin 1 (coding by SYNE1 gene) and nesprine 2 (coding by SYNE2 gene). Since it has previously been reported that in EDMD1 and in EDMD2 non-farnesylated prelamin A was mislocalized we investigated the distribution of such a protein precursor in EDMD fibroblasts harboring SYNE -1 and/or SYNE -2 genes mutations. In order to induce the accumulation of non-farnesylated lamin A precursor we treated EDMD fibroblasts with mevinolin, an inhibitor of 3-hydroxy-3-methylglutaryl-CoA reductase, that interferes with prelamin A farnesylation. The immunofluorescence detection of prelamin A and the electron microscopy analysis of treated cells clearly showed that the number and the distribution pattern of non-farnesylated prelamin A aggregates were altered. Moreover the accumulation of non-farnesylated lamin A precursor either in EDMD fibroblasts harboring LMNA gene mutations or in EDMD fibroblasts harboring SYNE 1 and 2 genes mutations was not able to induce the redistribution of heterochromatin marker trimethyl-K9-histone 3 at the prelamin A aggregates level as shown in control cells. These results suggest that non-farnesylated prelamin A localization and its effect on heterochromatin remodeling could be related to the nesprin/emerin/lamin interactions.

2078/B543

The Supramolecular Organization of the Nuclear Lamin Filaments.

K. Ben-Harush[1], N. Wiesel[2], D. Frenkiel-Krispin[1], D. Moeller[3], E. Soreq[4], U. Aebi[5], H. Herrmann[3], Y. Gruenbaum[2], O. Medalia[1]; [1]Life Science, Ben-Gurion University of the Negev, Beer-Sheva, Israel, [2]Genetics, Institute of Life Sciences, Hebrew University, Jerusalem, Israel, [3]Molecular Genetics, German Cancer Research Center, Heidelberg, Germany, [4]Animation, Bezalel Academy of Arts and Design, Jerusalem, Israel, [5]M. E. Mueller Institute for Structural Biology, Biozentrum, University of Basel, Basel, Switzerland

Nuclear lamins are involved in most nuclear activities and are essential for retaining the mechano-elastic properties of the nucleus. Mutations in human lamins cause laminopathic diseases, ranging from muscular dystrophies to lipodystrophies and premature aging. Lamins are nuclear intermediate filaments which form a distinct meshwork-like layer adhering to the inner nuclear membrane called the nuclear lamina. Here,

we present for the first time, the three-dimensional supramolecular organization of lamin 10 nm filaments and paracrystalline fibers using cryo electron tomography. We show that Caenorhabditis elegans nuclear lamin form 10-nm intermediate filaments (IF)-like lamin filaments that are distinct from their cytoplasmic counterparts. The IF-like lamin filaments are composed of 3 or 4 tetrameric protofilaments, each of which contains two approximately half-staggered anti-parallel head-to-tail polymers. The beaded appearance of the 10 nm IF-like lamin filaments stems from paired globular tail domains, which are regularly spaced, alternating between 21 and 27 nm. C. elegans lamin can also be assembled into paracrystalline fibers in vitro, when they assume a three-dimensional structure resembling that of the beaded IF-like filaments. Based on our structural analysis, we propose an assembly pathway that yields the observed 10 nm IF-like lamin filaments and paracrystalline fibers. These results serve as a platform for understanding the effect of laminopathic mutations on lamin supramolecular organization.

2079/B544

The Roles of Spectrin Repeat Proteins in Nuclear Envelope: Nesprin-1 A and A-II Spectrin.

Z. Zhong[1], K. L. Wilson[2], K. Dahl[1]; [1]Carnegie Mellon University, Pittsburgh, PA, [2]Department of Cell Biology, Johns Hopkins University, Baltimore, MD

The nuclear envelope consists of two membranes (INM and ONM), nuclear pore complexes, and an underlying protein structural support, the nuclear lamina network. In mammalian cells, the lamina is composed of A and B types of lamin proteins which recruit and anchor most INM proteins, including emerin, nesprin-1 α, LBR, et al. At least twelve disease syndromes have been linked to mutations in lamin A and lamin-binding proteins, termed as laminopathies. The mechanisms of laminopathies have not been determined, but current evidence is consistent with defects in tissue-specific gene expression coupled to mechanical weakness of the nuclear envelope. The nuclear lamins are stiff proteins which make up a majority of the nuclear lamina. Our previous work showed the nuclear lamina is not a stiff network but has 'spring-like' memory. Spectrins are a family of cytoskeleton proteins characterized with spring-like elastic structure. However, little is known about the spectrin roles in the nuclear envelope and its influence in nuclear lamina and laminopathies. Our work focuses on the roles of spectrin family proteins nesprin-1 α and α-II spectrin. We measured the thermal unfolding properties of several recombinant fragments of nesprin-1α and found that the central rod domain overstabilized the spectrin-like domains of nesprin-1α. Thus, despite having spectrin-like elements, we suggest the nesprin-1α is not the main contributor of the spectrin-like memory of nuclear lamina. The αII-spectrin is another nuclear spectrin protein. Our results suggest that αII-spectrin stabilizes laminopathy-related proteins emerin and lamina network, and it is an essential component of the nuclear lamina infrastructure and may contributes to the 'spring-like' memory of nucleus.

2080/B545

UNC-84, a C. Elegans Inner Nuclear Membrane SUN Protein Is Actively Imported into the Nucleus.

E. C. Tapley; Molecular and Cellular Biology, University of California Davis, Davis, CA

Nuclear positioning is important for a wide variety of developmental processes. Using C. elegans we have identified two conserved proteins that are required for nuclear migration. We propose that they function to bridge the nuclear envelope (NE), connecting the cytoskeleton to the nuclear matrix. Our model is that UNC-84 localizes to the inner nuclear membrane (INM) and that UNC-83 localizes to the outer nuclear membrane (ONM), with their respective SUN and KASH domains interacting in the perinuclear space (PNS) (McGee et.al 2006). Critical to refining our model is to determine UNC-84's topology and its retention at the INM. In vitro protease protection assays reveal a 65 kDa protected-fragment, consistent with UNC-84 having one TM domain located at residues 512-532. There are two current models on how INM proteins are localized: Diffusion-Retention and/or Active Import-nuclear localization signal (NLS) mediated. Consistent with both models, the N-terminal 535 residues of UNC-84, which contain 2 putative NLSs, are sufficient for NE localization in vivo. To test whether UNC-84 is actively imported we have expressed various UNC-84 GFP-LacZ fusion proteins in vivo. All GFP-LacZ fusion proteins are too large to enter the nucleus unless actively imported. UNC-84 1-370 is sufficient for NE localization. To test whether either of UNC-84's putative NLSs is functional, site directed mutagenesis was used to mutate each of two potential NLSs in our unc-84 rescuing construct. The double NLS mutant localizes to the NE. These experiments suggest that UNC-84 is actively imported and that neither putative NLS is required for its localization. In vivo localization studies of the other GFP-LacZ fusion proteins show that UNC-84 1-244 localizes to the cytoplasm while UNC-84 118-370 localizes to the NE. Taken with the above results, these findings suggest that: 1) UNC-84 contains an unidentified NLS between residues 244 and 370 or 2) that UNC-84 has an interacting domain within this region that allows it to piggy-back through the nuclear pore with an unidentified partner. Future work will address these questions, whether importin(s) are involved in UNC-84's active import, and if mammalian SUN proteins are also actively imported.

2081/B546

Protein-Free Liposomes Rescue Nuclear Envelope Formation by Fusion with Membrane Vesicles.

E. Rafikova, K. Melikov, C. Ramos, L. Dye, L. Chernomordik; National Institutes of Health, Bethesda, MD

In this study we explored relative contribution of membrane-residing proteins (MR, transmembrane proteins and other proteins that are very tightly associated with membranes and are not found in cytosol) and cytosolic proteins in nuclear envelope (NE) and endoplasmic reticulum (ER) assembly in cell-free system. Mixing of membrane vesicles (MVs) and cytosol isolated from Xenopus laevis egg extracts in the presence of sperm chromatin results in formation of nuclei with active DNA replication, functional nuclear pore complexes (NPCs) and expanded NE. Simultaneously branched network of ER is observed. We previously showed that protein-free dioleoyl phosphatidylcholine liposomes (LPs) fused with each other at the surface of chromatin in the presence of cytosolic proteins but failed to form nuclei (C. Ramos at al, 2006). In this study we replaced some of MVs with membrane vesicles functionally impaired by trypsin or N-ethyl maleimide treatments (MVs*) and with LPs. MVs* and LPs undergo cytosol- and GTP- dependent fusion with each other and with MVs, and participate in the formation of ER. In cytosol neither MVs* nor LPs form ER and functional nuclei, but they support assembly reactions by fusing with MVs. Nuclei that formed at lowered concentrations of MVs were smaller than the control ones, have decreased amounts of NPCs, and fail to actively replicate DNA and import substrates. Addition of LPs compensated for membrane shortage and restored the sizes of the nuclei. Furthermore, the liposome-rescued nuclei appear similar to the control ones in ultra-structure, active nuclear transport, DNA replication and

spatial distribution of NPC. The recovery of NE formation requires nuclear transport and involves GTP-dependent fusion between LPs and MVs and an increase in the number of functional NPCs. Our data demonstrate that a major change in its lipid composition and concentration of MR proteins has surprisingly little effect on function of NE and suggest that a feedback mechanism couples NE expansion with pore assembly.

2082/B547

Laminopathic Mutations Interfere with the Assembly, Localization and Dynamics of Nuclear Lamins in C. Elegans.

N. Wiesel Motiuk[1], A. Mattout[1], S. Melcer[1], N. Melamed-Book[1], H. Herrmann[2], O. Medalia[3], U. Aebi[4], Y. Gruenbaum[1]; [1]Genetics, Hebrew University, Jerusalem, Israel, [2]Cell Biology, DKFZ, Heidelberg, Germany, [3]NIBN, Ben-Gurion University, Beer Sheva, Israel, [4]M.E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Basel, Switzerland

Lamins are nuclear-specific intermediate filament proteins (IFs) that, together with a complex set of inner nuclear membrane proteins, form a filamentous meshwork between the inner nuclear membrane and the peripheral chromatin. Lamins are involved in most nuclear activities; and mutations in human lamins or their associated proteins cause laminopathic diseases ranging from muscular dystrophies to accelerated ageing. Previous studies showed that lamin dimers form filamentous structures through 'head-to-tail' interactions, which then form paracrystalline structures in vitro. C. elegans has a single evolutionarily conserved lamin protein, termed Ce-lamin. This lamin is unique in its ability to form under specific conditions 10-nm filaments, similar to other IFs. We have used negative staining to determine the structure of the 10-nm filaments and paracrystals and to find how mutations in residues, which are conserved in human lamin A and lead to laminopathic diseases, affect lamin filaments or paracrystals assembly. We have further analyzed the effects of these mutations on lamin organization and dynamics in vivo, by creating mutated gfp::lmn1 transgenic strains and by performing FRAP experiments. Our results show for the first time how mutations affect filament and paracrystal assembly in vitro and the localization and dynamics of Ce-lamin in vivo.

2083/B548

A Discrete Model for Mechanics, Transport, and Mechanotransduction in the Nuclear Pore Complex.

R. Moussavi Baygi, Y. Jamali, M. Mofrad; Molecular Cell Biomechanics, Department of Bioengineering, University of California, Berkeley, Berkeley, CA

Nuclear Pore Complex (NPC), a large macromolecular machine composed various kinds of nucleporins (Nups), is the only gateway to mediate nucleocytoplasmic transport. Although during past decade a significant progress has been made to explore the morphology and structural composition of NPC, still little is known about the actual transport mechanism. Amongst the most interesting features of transport mechanism are the massive rates of transportation of cargos and the intelligent selectivity of "big enough" cargos by NPC. We have modeled NPC as a dynamic, viscoelastic structure in a cytoplasmic environment incorporating the key components of the NPC structure. The model allows for investigation of the importance of FG repeats in transport mechanism. We have also made a comparison between some putative models. Our findings suggest that the NPC can be treated as a linear viscoelastic composite. FG repeats shorten during the cargo transverse and then extend again to obstruct the central channel, hence "non-permitted" particles cannot pass through, yet the enormous rate of nucleocytoplasmic transport can be observed. It seems that the randomly distributed of FG repeats have an important role in the transport selectivity offered by the NPC.

2084/B549

Structural Basis for Extended Modularity of the Nuclear Pore Complex.

S. Brohawn[1], N. C. Leksa[1], E. D. Spear[1], K. R. Rajashankar[2], T. U. Schwartz[1]; [1]Biology, MIT, Cambridge, MA, [2]Northeastern Collaborative Access Team, Argonne National Laboratory, Argonne, IL

Transport of macromolecules across the nuclear envelope is exclusively mediated by the nuclear pore complex (NPC). In order to fully understand the transport process, knowledge of the NPC architecture is key. However, at 40-60 MDa in size the NPC is one of the largest assemblies in the cell thus making it a formidable challenge to characterize structurally. Modularity within the NPC has been recognized for several years and can be exploited for structure determination following a divide-and-conquer approach. We have recently determined the crystal structure of the most abundant structural nuclear pore protein, Nic96. Together with the structure of a complex between two other scaffold proteins of the NPC, Nup107 and Nup133, we have now reanalyzed the phylogenetic relationships between the components of the NPC, with important conclusions for an Ur-NPC that preceded the extant form. This structure-based analysis allows us to substantially extend and specify the modularity concept of the NPC.

2085/B550

The N-Terminal Leucine Rich Repeat Functions to Anchor nup358 to the Nuclear Pore Complex.

E. Coutavas, G. Blobel; Cell Biology, Elias Coutavas, New York, NY

The nuclear pore complex (NPC) is the main aqueous transport route for macromolecular traffic between the nucleus and cytoplasm of eukaryotic cells. It spans the double bilayer of the nuclear envelope (NE) and is composed of ~30 different proteins named nucleoporins (nups), present in eight or, multiples of eight, copies. While the core nups are symmetrically localized, the cytoplasmic and nucleoplasmic faces of the NPC contain several uniquely localizing proteins. Little is known about the subunit interactions, stoichiometry and assembly/disassembly mechanisms governing the cytoplasmic filaments of the NPC. One of the nups at the cytoplasmic filaments is nup358 (1), a 358 kDa multi-domain protein that contains a ~900 amino acid N-terminal leucine-rich repeat region (LRR). We have used live cell imaging of HeLa cells expressing green fluorescent protein (GFP) fused to deletion mutants of the nup358 to map the NPC localization domain to the entire LRR and not smaller subdomains. By fluorescence recovery after photobleaching we have determined that this domain remains anchored to the NPC during interphase, is released from the NPC to a soluble pool during NE breakdown, and reassembles at the NPC in late telophase. We have begun to identify interaction partners of nup358 at the NPC by immunoaffinity purification with anti-GFP antibodies and mass spectrometry. (1) Wu J. et al. (1995), JBC 270(23):14209-13

2086/B551

Regulation of Barrier-to-Autointegration Factor (BAF) Function through Phosphorylation.

J. Lei[1], M. Wiebe[2], L. O'Brien[3], C. Wiese[3], P. Traktman[2], M. Segura-Totten[1]; [1]Biology, The College of New Jersey, Ewing, NJ, [2]Microbiology and Molecular Genetics, Medical College of Wisconsin, Milwaukee, WI, [3]Biochemistry, University of Wisconsin-Madison, Madison, WI

Barrier-to-Autointegration Factor (BAF) is a conserved DNA and LEM domain binding protein with roles in nuclear assembly. BAF interaction with LEM proteins and DNA is necessary for the proper organization of the nuclear envelope upon nuclear reformation. Phosphorylation of Ser-4 and Thr-2/3 by the vaccinia-related kinase 1 (VRK1) abolishes BAF interaction with DNA and lowers the affinity of BAF for LEM proteins. Additionally, BAF is hypophosphorylated and constitutively localized to chromatin in VRK-depleted *C. elegans* embryos. We hypothesize that differential phosphorylation of BAF regulates its DNA and LEM domain binding activities *In Vivo*, allowing BAF to release membranes at the start of mitosis and enabling BAF to recruit membranes to chromatin at the end of mitosis. We also hypothesize that phosphorylation of BAF at the telophase-interphase junction allows for chromatin decondensation, nuclear expansion and growth. To test our hypotheses, we added BAF proteins mutated at Ser-4 and Thr-2/3 to *Xenopus laevis* nuclear assembly reactions. Nuclei assembled with a BAF protein mutated in all three residues are growth-arrested and have condensed chromatin. Addition to nuclei of BAF proteins with an aspartic acid substitution at Ser-4 that mimics phosphorylation leads to enhanced chromatin decondensation and nuclear growth. These findings suggest that phosphorylation of BAF regulates chromatin decondensation and nuclear growth. A BAF protein mutated at Thr-2/3 exhibits a loss of the enhanced chromatin decondensation and nuclear growth activities of wild type BAF, suggesting that phosphorylation at these residues regulates BAF function during nuclear assembly. However, these residues are not phosphorylated in interphase *Xenopus* extracts. We are currently determining the phosphorylation state of these residues during nuclear assembly. TEM of nuclei incubated with high concentrations of BAF proteins mutated at Ser-4 and Thr-2/3 reveal chromatin that is strikingly different from nuclei assembled with high concentrations of wild type BAF. Our results suggest that Thr-2/3 and Ser-4 are important in regulating the effect of BAF on chromatin structure, as well as BAF-mediated chromatin decondensation and nuclear growth.

2087/B552

LULL1 Retargets Active Torsina to the Nuclear Envelope, Where It Displaces the Inner Nuclear Membrane Protein Sun2.

A. L. Buchwalter[1], T. V. Naismith[1], D. Hodzic[1], E. Snapp[2], P. I. Hanson[1]; [1]Cell Biology and Physiology, Washington University in St. Louis, St Louis, MO, [2]Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

The Torsin subfamily of AAA+ ATPases resides within the lumen of the endoplasmic reticulum (ER) and contiguous nuclear envelope (NE). TorsinA (TorA) is mutated in *DYT1* dystonia, but its normal function and the basis for disease remain unclear. TorA has been shown to bind the ER-localized protein, LULL1. Here, we report the surprising ability of overexpressed LULL1 to promote redistribution of TorA from the bulk ER to the NE. As TorA concentrates in the NE, it displaces the LINC (linker of the nucleoskeleton and cytoskeleton) complex component Sun2, but not the related Sun1, from the NE. Over time, redistributed TorA perturbs NE organization leading to expanded nuclear membranes and an irregular perinuclear space. LULL1-driven targeting of TorA to the NE requires that the enzyme's ability to bind and hydrolyze ATP is intact, and also that an N-terminal hydrophobic domain and conserved cysteine residues are present. These data point to a critical role for the N-terminal domain in targeting TorA to specific regions within the ER system. The dystonia-causative ΔGAG-TorA mutant is as previously reported inherently enriched in the NE, but it too can be further concentrated in the NE by coexpression with LULL1. Knock-down of LULL1 further suggests that LULL1 is responsible for the normal steady-state enrichment of ΔGAG-TorA in the NE. Interestingly, LULL1-redistributed ΔGAG-TorA displaces Sun2 from the NE more slowly and has less effect on NE structure than does the wild-type enzyme. The ability of LULL1 to target TorA to the NE provides insight into how TorA may preferentially affect proteins within a subdomain of the ER network. Further, our findings indicate that control of both TorA localization and activity may be compromised by the dystonia-associated ΔGAG deletion.

2088/B553

A Quantitative Kinetic Analysis of Nuclear Envelope Formation.

J. Vargas[1,2], D. Anderson[1,2], M. Hetzer[1], J. P. Hsiao[1]; [1]Molecular and Cell Biology Laboratory, The Salk Institute for Biological Studies, La Jolla, CA, [2]Biology, University of California, San Diego, La Jolla, CA

Nuclear envelope (NE) formation is a complex process that involves a massive reorganization of the endoplasmic reticulum (ER). We have previously demonstrated that ER membrane tubules are recruited to and flatten on DNA in the absence of cytosolic proteins, suggesting a role for integral membrane proteins in NE formation. In order to investigate the contribution of transmembrane proteins, we have developed an in vivo assay that allows for the accurate quantification of the time required for NE formation. Our data suggests that reduced levels of several INM proteins delay NE formation without disrupting interphase nuclear morphology. The ability of a ~90% reduction in certain INM proteins to slow, but not prevent, NE formation suggests redundancy in their function. Surprisingly, over-expression of several INM proteins (e.g. LBR, Lap2β) accelerates NE formation, an apparent increase in the efficiency of the process. Further, analysis of hLap2β protein fragments supports a role for INM proteins where a physical bridge between membranes and chromatin is formed through INM protein/chromatin interactions. High-resolution imaging demonstrates that flattening of membranes around chromatin is the key step that is modulated by INM proteins. The function of INM proteins can be disconnected from lamina assembly as reductions in lamin protein levels has only marginal effects on NE formation. These results suggest a model for NE formation driven by an additive accumulation of protein-chromatin interactions that facilitate recruitment of ER tubules onto the mitotic chromatin surface.

2089/B554

E145K Mutant Lamin a Leads to Defects in Chromosome Segregation in Progeria, a Premature Aging Syndrome.

P. Taimen, K. Pfleghaar, R. Goldman; Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL

The premature aging disease Hutchinson-Gilford Progeria Syndrome (HGPS) is typically caused by a single point mutation (1824 C>T) in the human nuclear lamin A (LA) gene (*LMNA*), resulting in the synthesis of LA with 50 amino acids deleted near its C terminus (LAΔ50). In

rare cases, a point mutation in the rod domain of LA (e.g. E145K) has been connected to HGPS but to date the cellular mechanism leading to this form of the disease has not been analyzed. We have investigated the impact of the E145K-LA mutation on overall nuclear shape and chromatin organization. Fibroblasts from patients with the E145K mutation showed severe nuclear lobulation in early passages and the mislocalization of heterochromatin marks, nuclear pores, centromeres, RNA polymerase II, as well as the A- and B-type lamins. Transfection of HeLa cells with FLAG-tagged E145K-LA induced nuclear lobulations and mislocalization of nuclear proteins similar to those changes seen in patient fibroblasts. These changes were typically seen in postmitotic cells and correlated with post-transfection time suggesting that E145K-LA interferes with nuclear reassembly in daughter cells and an inability to reorganize chromatin in early G1. Supported by a Grant from the NIA, a Senior Scholar Award from the Ellison Foundation, the Sigrid Juselius Foundation and the Deutsche Forschungsgemeinschaft.

2090/B555

In Vitro Assembly Schedules for Recombinant Human A-Type Lamins and Selected Disease Mutants.

H. Herrmann[1], D. Moeller[1], N. Mücke[2], U. Aebi[3]; [1]Functional Architecture of the Cell B065, German Cancer Research Center (DKFZ), Heidelberg, Germany, [2]Biophysics of Macromolecules B040, German Cancer Research Center (DKFZ), Heidelberg, Germany, [3]M. E. Müller Institute for Structural Biology, Biozentrum, University of Basel, Basel, Switzerland

In order to find new ways for the directed assembly of lamins, we have investigated the range of ionic conditions that allow to keep A-type lamins in a distinct soluble state. For this purpose, recombinant lamins were solubilized in 8 M urea and purified by column chromatography. Renaturation was by step-wise dialysis into 20 mM Tris, pH 9.0, 500 mM sodium chloride. Starting from this condition, we explored to what extent the salt concentration could be reduced such that homogeneous soluble dimeric complexes were still obtained. In addition, we explored the use of several buffer substances in place of Tris. The degree of solubility of lamin A at the various stages of renaturation was analyzed by low speed centrifugation followed by gel electrophoresis of the soluble and sedimented fractions. At 20 mM Tris-HCl, pH 7.5, and 200 mM NaCl we still encountered no significant loss of lamin A. However, after further reduction of the salt concentration in 10 mM steps, the protein was progressively and massively lost indicating association of soluble subunits to higher order complexes such that at 150 mM NaCl most lamin A was pelletable. By analytical ultracentrifugation we revealed sedimentation coefficients of around 3.9, 4.1 and 4.4 S for the soluble complexes at pH 9.0 plus 500 mM NaCl, pH 7.5 plus 500 mM NaCl and pH 7.5 plus 250 mM NaCl, respectively. This indicated a salt-induced structural reorganization of the dimers as a result of new ionic interactions of the tail domains with the rod domains. Starting from these latter conditions, we followed by transmission electron microscopy of negatively stained samples the assembly of lamins induced by the reduction of the salt concentration through addition of distinct volumes of salt-free buffer. Here we observed dramatic differences in the filamentous structures formed at sodium chloride concentrations as close as 190 mM and 175 mM NaCl indicating prominent differences in the intra- and inter-molecular interactions when the protein is challenged with changes in the ionic condition at near-physiological values. This may reflect the fact that lamin C is missing 90 amino acids in its tail domain present in lamin A.

2091/B556

Effect of Nuclear Envelope Proteins on Cytoskeletal Structure and Function.

M. Lombardi[1], C. Shanahan[2], J. Lammerding[1]; [1]Department of Medicine, Brigham and Women's Hospital, Harvard Medical School, Cambridge, MA, [2]Cardiovascular Division, King's College London, London, United Kingdom

Mutations in the nuclear envelope proteins lamins A and C cause a number of human diseases, including Emery-Dreifuss muscular dystrophy, limb-girdle muscular dystrophy, and dilated cardiomyopathy. The molecular mechanisms underlying the muscle specific defects in these diseases remain unclear. The recent discovery of two nuclear membrane protein families, nesprins and SUN proteins, may now provide an additional facet to these diseases. Nesprins and SUN proteins form the LINC complex, physically linking the nucleus to the cytoskeleton. Lamins A and C may contribute to the localization of these proteins to the nuclear envelope, thus altering the LINC complex function. To investigate the effect of nuclear envelope proteins on nuclear-cytoskeletal coupling, we have designed novel experimental methods to probe LINC complex function in lamin A/C-deficient mouse embryo fibroblasts, wild-type controls, and in cells in which the LINC complex has been disrupted by dominant negative mutants or RNA interference. By measuring the diffusional mobility of GFP-nesprin constructs at the nuclear envelope by fluorescence recovery after photobleaching, we found that nesprins have increased mobility at the nuclear envelope compared to lamin A. To provide functional evidence that the LINC complex is necessary for force transmission from the cytoskeleton to the nucleus, we have designed a novel approach that measures induced nuclear deformation in response to applied cytoskeletal and nuclear strain. We observed reduced nuclear deformations in lamin A/C-deficient mouse embryo fibroblasts compared to wild-type cells during applied cytoskeletal strain. Our studies imply that mutations in nuclear envelope proteins could generally contribute to the development of muscular dystrophies by disrupting nuclear-cytoskeletal coupling and disturbing the mechanical and functional properties of the nucleus and cytoskeleton.

2092/B557

Dissecting the Role of POM121 in Nuclear Pore Complex Assembly and Function.

J. Talamas, M. Hetzer; The Salk Institute for Biological Studies, La Jolla, CA

Nuclear pore complexes (NPCs), the exclusive sites of nucleo-cytoplasmic transport, reside in the nuclear envelope (NE) of eukaryotic cells. These giant 125 MD protein complexes are composed of approximately 30 known proteins, called nucleoporins (nups), each present in multiples of eight. Three of the nups, POM121, Ndc1 and gp210, are transmembrane proteins. POM121, unique to metazoans, has been shown to be essential for NPC assembly by siRNA knockdown in HeLa cells and by depletion from Xenopus extracts. NPC assembly has been shown to initiate with recruitment of the Nup107/160 complex to chromatin via ELYS. The mechanisms of subsequent recruitment of additional nups and the NPCs incorporation into the double lipid bilayer of the NE are currently unknown. Due to its requirement for NPC assembly, transmembrane nature and large nucleoplasmic domain POM121 is postulated to mediate the assembling NPCs connection to the NE. We are using structure-function analysis and protein dynamic studies to identify functional domains of POM121. We have identified the minimum protein domains of POM121 for targeting to the NE and separately for NPC association. We are currently investigating protein-

protein interactions to identify specific and novel interacting partners of POM121. Our data suggests that interactions with both the NE proteins and components of the NPC are important for POM121 incorporation and function.

2093/B558

Regulation of Lamin B1 by mIR-23 Is Critical for Normal Oligodendrocyte and Myelin.

S. Lin, Y. Fu; Department of Neurology, University of California San Francisco, San Francisco, CA

Duplication of LMNB1 with increased mRNA and protein levels has been shown to cause severe myelin loss in the brains of adult-onset autosomal dominant leukodystrophy patients. Similar to many neurodegenerative disorders, patients with adult-onset autosomal dominant leukodystrophy are phenotypically normal until adulthood and the defect is specific to central nervous system despite the ubiquitous expression pattern of lamin B1. We set out to dissect the molecular mechanisms underlying this demyelinating phenotype. Increased lamin B1 expression results in disturbances in inner nuclear membrane protein, chromatin organization, and nuclear pore transport. It also leads to premature arrest of oligodendrocyte differentiation, which may be caused by reduced transcription of myelin genes and by mislocalization of myelin proteins. We identified miR-23 as a negative regulator of lamin B1 that can ameliorate the consequences of excessive lamin B1 at the cellular level. Our results indicate that regulation of lamin B1 is important for myelin maintenance and miR-23 contributes to this process at least in part by downregulating lamin B1 therefore establishing novel functions of lamin B1 and miR-23 in regulation of oligodendroglia development and myelin formation.

2094/B559

Characterization of Nesprin-1 Function at the Nuclear Envelope Characterization of Nesprin-1 Function at the Nuclear Envelope.

S. Taranum[1,2,3], W. Lu[4], I. Karakesisoglou[4], A. A. Noegel[1,2,3]; [1]Center for Biochemistry, Medical Faculty, University of Cologne, Cologne, Germany, [2]International School in Genetics and Functional Genomics, University of Cologne, Cologne, Germany, [3]Cellular Stress Responses in Aging-Associated Diseases, University of Cologne, Cologne, Germany, [4]School of Biological and Biomedical Sciences, Durham University, Durham, United Kingdom

Nesprin-1 is a giant nuclear envelope associated actin-binding protein belonging to the spectrin superfamily. The largest predicted Nesprin-1 isoform (Nesprin-1 Giant/Enaptin; 1086kDa) exhibits an N-terminal actin-binding domain followed by a long spectrin repeat containing rod and a C-terminal type II transmembrane domain which is sufficient to anchor this protein to the nuclear membrane. In order to confirm the existence of Nesprin-1 Giant we performed western blot analysis on several cell lines using antibodies specific to the N- and C-terminus of Nesprin-1. Our study shows that Nesprin-1 Giant is expressed in detectable levels in differentiated mouse myoblast and human glioblastoma cell lines. Immunofluorescence microscopy showed upregulation of Nesprin-1 during myoblast differentiation. We also report that the overexpression of Nesprin-1 KASH domain results in the mislocalization of emerin and loss of Nesprin-1, Nesprin-2 and LAP2 at the nuclear envelope. Preliminary data suggest that Nesprin-1 KASH overexpression may also result in a cytokinesis defect. Nesprin-3 was shown to interact with MACF1 and plectin actin-binding domains in vitro (Wilhelmson et.al., 2005). We provide evidence that Nesprin-3 also interacts with the actin binding domains of Nesprin-1 and-2 and recruits them to the nuclear envelope. This implies the existence of a filamentous network of Nesprins along the outer nuclear membrane suggesting a role for Nesprins in the maintenance of nuclear shape and integrity.

2095/B560

The Role of Nups in Mitosis: a Systematic Rnai Screen in Drosophila Cells.

S. A. Ribeiro, A. R. Martins, C. Chandsawangbhuwana, R. D. Vale, E. R. Griffis; Physiology Course, Marine Biological Laboratory, Woods Hole, MA

The nuclear pore complex (NPC) is a large proteinaceous structure composed of multiple copies of 30 proteins termed nucleoporins (Nups). During interphase, the NPC's main role is the bidirectional transport of soluble molecules between the nucleus and cytoplasm. In higher eukaryotes, entry into mitosis is coincident with nuclear envelope breakdown, NPC disassembly and dispersion of Nups. Recent studies have demonstrated that some Nups have functions in mitosis. In order to elucidate the role of nucleoporins in mitosis we carried out a comprehensive RNAi screen in S2 cells. We performed automated live and fixed analysis using S2 cells expressing mCherry-tubulin and H2B-GFP. Of the 29 dsRNAs used, 5 showed mitotic index alterations, spindle morphology and/or chromosome alignment defects. Further live cell imaging of the positive hits was carried out using S2 cells expressing Lamin B-GFP, Mis12-GFP, or Mad2-GFP together with mCherry-tubulin. Two of the nucleoporins, Nup133 and Nup43, belong to the well characterized Nup107-160 complex. Depletion of Nup133 caused chromosome alignment defects and mitotic delay. Nup43-depleted cells also showed a mitotic delay, accompanied by a defect in recruitment of Mad2 to kinetochores. Depletion of two other Nups - Nup205 and Nup50 - which have no described localization in mitosis, showed defects in anaphase-B spindle elongation. Finally, ALADIN-depleted cells display chromosome alignment and segregation defects. This systematic study of all Nucleoporins in Drosophila cells led us to find 3 Nups that have novel roles in chromosome alignment and segregation. Further experiments are underway to characterize the functions of these proteins in mitosis, and to rescreen all Nups for other roles in mitosis.

Blood Vessels (2096 – 2107)

2096/B562

Temporal Analysis of Proteomics on Balloon Injury-Induced Neointima Formation.

C. Wu[1], M. Sung[2]; [1]Pharmacy, China Medical University, Taichung, Taiwan, [2]Medicine, China Medical University, Taichung, Taiwan

There have been known approximate 30 % to 50 % of the patients receiving a successful percutaneous transluminal coronary angioplasty (PTCA) suffered from restenosis by six months. Though stents have been widely used to decrease the incidence of restenosis, yet 20 % to 30 % of the patients are still affected by restenosis after coronary stenting. Currently, there are no valid markers to elucidate the pathological process and thus the development of a therapeutic remedy. In the current study, we aimed to explore the temporal pathologies of protein

profiles in the injured vessels at 2 hours, 2 days, 7 days, and 14 days after the angioplasty. Our results demonstrated the neointimal formation at the beginning of 7 days and becoming significant at 14 days after angioplasty. Thereafter, the protein profiles from the injured vessels at various time points were analyzed by 2D-GE. Four proteins have been identified by Mass Spectrometry to be closely associated with neointimal formation in a time-dependent manner. They were disulfide-isomerase A3 precursor (PDIA3), glutathione S-transferase, pi2 (GSTp2), glutamate dehydrogenase 1 (GLUD1), and phosphoglycerate kinase 1(PGK1). These four proteins and their associated molecules were then confirmed by Western blot. These observations indicate that PDIA3, GSTp2, GLUD1, PGK1 are importantly involved in the process of neointimal formation, suggesting their potential roles as biomarkers and/or therapeutic targets for restenosis.

2097/B563

Thrombin-Induced COX-2 Expression via C-Src/PKC-Mediated EGF Receptor Transactivation in Vascular Smooth Muscle Cells.

C. Yang, H. Hsieh, C. Sun; Pharmacology, Chang Gung University, Tao-Yuan, Taiwan

The thrombin/proteinase-activated receptors (PARs) have been shown to regulate smooth muscle cell proliferation, migration, and vascular maturation. Thrombin up-regulates expression several proteins including cyclooxygeanse (COX)-2 in vascular smooth muscle cells (VSMCs) and contributes to vascular diseases. However, the mechanisms underlying thrombin-regulated COX-2 expression in VSMCs remain unclear. Western blotting, RT-PCR, and EIA kit analyses showed that thrombin induced the expression of COX-2 mRNA and protein and PGE2 release in a time-dependent manner, which was attenuated by inhibitors of PKC (GF109203X, Gö6976, and rottlerin), c-Src (PP1), EGF receptor (EGFR; AG1478) and MEK1/2 (U0126), or transfection with dominant negative mutants of PKC-δ, c-Src or extracellular regulated kinase (ERK) and ERK1 short hairpin RNA interference (shRNA). These results suggest that transactivation of EGFR participates in COX-2 expression induced by thrombin in VSMCs. Accordingly, thrombin stimulated phosphorylation of ERK1/2 which was attenuated by GF109203X, PP1, AG1478, or U0126, respectively. Furthermore, this up-regulation of COX-2 mRNA and protein was blocked by selective inhibitors of AP-1 and NF-κB, curcumin and helenalin, respectively. Moreover, thrombin-stimulated activation of COX-2 promoter activity was blocked by the inhibitors of c-Src, PKC, EGFR, MEK1/2, AP-1 and NF-κB, suggesting that thrombin induces COX-2 promoter activity mediated through c-Src/PKC-dependent EGFR transactivation, MEK-ERK1/2, AP-1, and NF-κB. These results demonstrate that in VSMCs, activation of ERK1/2, AP-1 and NF-κB pathways was essential for thrombin-induced COX-2 gene expression. Understanding the regulation of COX-2 expression and PGE2 release by thrombin/PARs system on VSMCs may provide potential therapeutic targets of vascular inflammatory disorders including arteriosclerosis.

2098/B564

The C-Type Lectin-Like Domain of Thrombomodulin Interacts with Lewis Y Antigen and Inhibits Angiogenesis Induced by EGF.

C. Kuo[1,3], C. Shi[2,3], B. Chang[2,3], M. Sung[2,3], H. Wu[2,3], G. Shi[2,3]; [1]Institute of Basic Medical Sciences, National Cheng Kung University, Tainan, Taiwan, [2]Department of Biochemistry and Molecular Biology, National Cheng Kung University, Tainan, Taiwan, [3]Cardiovascular Research Center, National Cheng Kung University, Tainan, Taiwan

Thrombomodulin (TM) is a type-I glycoprotein on vascular endothelial cell surface, and serves as a thrombin receptor to mediate protein C dependent anticoagulation system. Beyond membrane tethered TM, several TM fragments in human plasma, urine and synovial fluid have been detected in physiological and various pathological conditions. However, the physiological significance of these TM fragments is still unclear. To study the functions of these fragments, three recombinant TM domain proteins were prepared and their biological functions were investigated. The results showed that ecto-domain of TM lacking the lectin-like domain had higher angiogenic activity than the whole ecto-domain. The lectin-like domain of TM alone (TMD1) inhibited endothelial cell activation, proliferation, migration and differentiation elicited by epidermal growth factor (EGF) in vitro. Furthermore, the inhibitory effect of TMD1 on EGF-elicited endothelial cell chemotaxis was abolished by multivalent Lewis Y antigen. The interactions of TMD1 with Lewis Y antigen and EGF receptor were demonstrated. TMD1 effectively inhibited EGF-mediated angiogenesis in the murine Matrigel model. The results suggested that TMD1 can directly interact with Lewis Y antigen and interfere with EGF receptor downstream signals, thereby inhibiting angiogenesis. We concluded that TMD1 represents a new type of anti-angiogenic factor. It might have therapeutic potential for treatment of different diseases involving neovascularization.

2099/B565

Transforming Growth Factor β and Bone Morphogenetic Protein Receptor II Ligands Regulate Elastin Synthesis via Peroxisome Proliferator-Activated Receptor β.

T. Kleisli[1,2], T. Alastalo[2], V. de Jesus Perez[3], M. Rabinovitch[2]; [1]Pediatric Cardiac Surgery, Stanford University, Stanford, CA, [2]Pediatric Cardiology, Stanford University, Stanford, CA, [3]Pulmonary Critical Care, Stanford University, Stanford, CA

Introduction: Children with familial supravalvular aortic stenosis (SVAS) or Williams syndrome (WS) develop vascular stenoses due to elastin haploinsufficiency. Therapies promoting elastin synthesis may therefore prevent these vasculopathies. Transforming growth factor beta (TGF-β) can be anti-proliferative in smooth muscle cells (SMC), consistent with its ability to induce elastin, which also inhibits SMC growth. Our laboratory has shown that bone morphogenetic proteins (BMPs) can inhibit human pulmonary artery (hPA) SMC proliferation in a manner dependent on peroxisome proliferator-activated receptor γ (PPARγ), but whether they upregulate elastin is unknown. Objective: We hypothesized that TGF-β and BMPs induce elastin via PPARγ. Methods: Primary central vessel hPASMC were stimulated with TGF-β and BMPRII ligands (BMP-2, -4, and -7) and elastin mRNA levels measured by qRT-PCR. To assess PPARγ role in mediating this response, we used the PPARγ agonist, Rosiglitazone (1uM), and antagonist, GW9662 (1uM). Results: Dose response (0, 12 and 24 hours (h) versus control) with TGF-β (0.1, 1, and 10ng/mL) and BMPs (1, 10, and 100ng/mL), yielded a 2.5-fold increase in elastin mRNA for TGF-β (maximum at 10ng/mL ($p<0.001$)), and a 2-fold, 15-fold and 4-fold increase for BMP-2, -4, and -7, respectively (beginning at 10, but maximum at 100ng/mL ($p<0.001$)). Time course (0, 4, 8, 12 and 24h versus control) with TGF-β (2ng/mL) and BMPs (10ng/mL) revealed peak elastin mRNA levels at 12h for TGF-β (2-fold) and at 24h for BMP-2 (1.5-fold), BMP-4 (5-fold) and BMP-7 (2-fold) ($p<0.001$, respectively). Treatment with Rosiglitazone or GW9662 did not affect unstimulated elastin mRNA levels. However, one hour pre-treatment with GW9662 followed by stimulation with TGF-β or BMPs, led to repressed increases in elastin mRNA levels for TGF-β (1.5-fold) and

BMP-4 (2-fold), and inhibition for BMP-2 and BMP-7 ($p<0.001$, respectively). Conclusions: TGF-β and BMPRII ligands increase elastin mRNA levels in hPASMC, suggesting shared functions in vascular regeneration. This may occur via a common PPARγ mechanism, inducing elastin synthesis while reducing hPASMC growth. Targeting this pathway may benefit patients with SVAS and WS.

2100/B566

Mechanical Strain Activates a Program of Genes Functionally Involved in Paracrine Signaling of Angiogenesis in the Bladder.

R. Yang, H. Liu, B. Chaqour; Dept of Anatomy and Cell Biology, SUNY, Brooklyn, NY

To enhance its load bearing potential, the urinary bladder elicits adaptive responses acutely through hypertrophy and rapid growth and chronically by remodeling the extracellular matrix. This tissue remodeling is a tenuous compromise requiring concomitant development of new blood vessels to meet higher metabolic demands. Studies were performed to examine the extent to which mechanical stimuli mediate control of angiogenesis in bladder cells both in vitro and in vivo. Differential gene expression between control non-stretched and cyclically stretched bladder smooth muscle cells was assessed using oligonucleotide microarrays and pathway analysis by the web tool Fast Assignment and Transference of Information (FatiGO). Data showed that a substantial proportion (33 of 86) of mechanically responsive genes were angiogenesis-related and include cytokines, growth-related factors, adhesion proteins, and matricellular, signal transduction, extracellular matrix (ECM) and inflammatory molecules. Integrative knowledge of protein-protein interactions revealed that 12 mechano-sensitive gene-encoded proteins have interacting partner(s) in the vascular system confirming their potential role in paracrine regulation of angiogenesis. Angiogenic genes include matricellular proteins such as Cyr61/CCN1, CTGF/CCN2 and tenascin C, components of the VEGF and IGF systems, ECM proteins such as type I collagen and proteoglycans and matrix metalloproteinases. In an in vivo model of bladder overdistension, 5 of 11 mechano-responsive angiogenic genes, independently tested by real-time PCR, were upregulated as a result of pressure overload including Cyr61/CCN1, CTGF/CCN2, MCP-1, VEGF-A, MMP-1 and midkine. Meanwhile, the molecular anatomy of angiogenic gene promoters reveals the presence of GA box-binding for the myc-associated zinc finger protein, MAZ, often found adjacent to binding sites for mechano-responsive transcription factors (e.g., NF-kB), suggesting that the coordinated activity of these factors may induce selective angiogenic gene transcription. These data suggest that mechanical control of angiogenic genes is an integral part of the adaptive and plasticity responses to mechanical overload.

2101/B567

Collagen-Induced Oxidation of SHP-2 Promotes Platelet Activation.

J. Jang[1], J. Min[1], Y. Kim[2], Y. Noh[1], J. Baek[1], H. Shon[1], Y. Yang[2], T. Chang[1,2,3]; [1]Life and Pharmaceutical Sciences, Ewha Womans University, Seoul, South Korea, [2]Molecular Life Science and Technology, Ewha Womans University, Seoul, South Korea, [3]College of Pharmacy, Ewha Womans University, Seoul, South Korea

The generation of reactive oxygen species (ROS) is observed in stimulated platelets. It has been proposed that generated ROS may act as second messengers in adhesion and aggregation of platelets, but direct mechanism of action of ROS in support of this hypothesis is still lacking. We then studied the cellular signaling network through which ROS amplify the aggregatory response of platelets. One redox-sensitive target that regulates signaling is the family of protein tyrosine phosphatases (PTPs), which have an oxidation-sensitive and active cysteine. Here, we show that antioxidants results in the inhibition of collagen-stimulated platelet aggregation and the increase in the rate of tyrosine dephosphorylation after time to reach the maximal level. We observed that activation of platelets by collagen induces production of ROS and oxidation of catalytic cysteine residue of the SH2 domain-containing PTP, SHP-2. To gain an insight into the critical role of H2O2 and its target SHP-2 during platelet activation, we used platelets obtained from mice lacking combinations of peroxidase, catalase and glutathione peroxidase 1 (GPx1). Catalase/GPx1 deficiency results in increased production of H2O2, enhanced oxidation and inactivation of SHP-2, and subsequently increased platelet activation in respond to collagen. As a consequence, in a model of carotid artery injury, thrombosis was markedly accelerated in Catalase/GPx1-deficient mice. Together, our findings suggest that collagen-induced ROS generation leads to SHP-2 oxidation, which promotes platelet activation, and implied a novel peroxidase-dependent inhibitory mechanism regulating arterial thrombosis.

2102/B568

Thrombin- Derived Peptide, TP508, Induces Vasorelaxation in Isolated Rat Aortic Tissue.

C. Dreiza, M. Sheller; Research Department, OrthoLogic Corporation, Tempe, AZ

Endothelial nitric oxide synthase (eNOS) is the constitutively active enzyme in endothelial cells that converts arginine to nitric oxide. Stimuli that inhibit eNOS upregulation contribute to endothelial dysfunction, which can be characterized by impaired vasorelaxation. Recently, thrombin has been shown to stimulate Ser1177 phosphorylation and modulate eNOS activity in human umbilical vein endothelial cells (FEBS Letters 573:175-180, 2004). Thus, the thrombin-derived synthetic peptide TP508 may prove beneficial as a therapeutic to ameliorate endothelial dysfunction through its potential action on eNOS activity. The aim of this study was to investigate the vasorelaxing potential of TP508 on isolated smooth muscle tissue using a muscle bath system. In the initial experiments, rat aortic rings (endothelium intact) were pretreated with or without TP508, contracted with norepinephrine, then treated with increasing doses of carbachol. In subsequent experiments, rat aortic rings (endothelium intact) were contracted with norepinephrine then treated with increasing doses of TP508 or carbachol. Experimental data demonstrated that (i) TP508 pretreatment enhanced carbachol-induced relaxation and (ii) TP508 inhibited norepinephrine-induced vasocontraction in a statistically significant manner compared to controls. Although additional work is required, this preliminary data suggest that TP508 could function via eNOS to induce relaxation of isolated rat aortic rings. Hence, tissue in which eNOS activity is attenuated may be a therapeutic target for TP508.

2103/B569

Ambulatory Function Is Preserved and Vessel Density Elevated Following Surgically-Induced Unilateral Hindlimb Ischemia in Cx37-Deficient Mice.

J. S. Fang, S. N. Angelov, J. M. Burt, A. M. Simon; Physiology, The University of Arizona, Tucson, AZ

Connexin37 (Cx37) and Connexin40 (Cx40) are endothelial gap junction proteins that produce similarly cation-selective channels. Yet, based on differences in vascular phenotype observed in their respective knockout animals, these connexins likely fulfill distinct (though overlapping) roles in the vascular endothelium. In in vivo settings where vascular remodeling and angiogenesis are stimulated, Cx37 expression is often down-regulated; in some in vitro settings Cx37 (but not Cx40) expression exerts a profound growth-suppressive effect by drastically prolonging cell cycle length. These and other previously reported findings suggest that while Cx37 and Cx40 form channels with similar permselective properties, they may nevertheless exert unique regulatory effects on the vascular remodeling process. Here, we use the previously established unilateral hindlimb ischemia model (wherein the femoral and saphenous artery/vein pair is resected in the surgical hindlimb) to produce ischemia affecting the entire hindlimb downstream of the resection and induce rapid vascular remodeling and angiogenesis in the affected tissue. We report that Cx37-deficient animals, compared to wild-type controls, experience less severe impairment of ambulatory function at all time points examined throughout the entire 14-day recovery period. Cx37-deficient animals also show an elevated vessel density in the gastrocnemius muscle of the ischemic hindlimb at 3, 7, and 14-day timepoints, differences that are unexplained by changes in muscle weight or muscle fiber size. Our results suggest that Cx37 represents an important inhibitory regulator of vascular remodeling that normally facilitates remodeling to an appropriate vascular density. Further in wild-type animals, under conditions that stimulate angiogenesis, Cx37 expression may be temporarily downregulated to relieve Cx37's growth suppressive signal, thereby allowing reparative vascular remodeling to occur. Our findings strongly suggest that novel therapies for treatment of ischemia-related tissue damage or disease could be developed targeting Cx37 expression and/or function. Supported by: HL064232 (AMS), AHA 550158Z (JMB) and AHA0715532Z (JSF).

2104/B570

Ca2+ Imaging Revealed Regional Differences of 5-HT Receptors in Cerebral and Testicular Arterioles of Rats: Dependence on Arteries Size and Organ Specificity.

K. Masu[1,2], T. Saino[2], K. Ogasawara[1], Y. Satoh[2]; [1]Neurosurgery, Iwate Medical University, Morioka, Japan, [2]Anatomy (cell biology), Iwate Medical University, Morioka, Japan

[Objective] Control of arterioles can be essential in tissue circulations of many organs, although the knowledge of physiological responses of arterioles was limited. In a previous study, we examined the intracellular calcium ion ([Ca2+]i) dynamics of arterioles isolated from rat brain and testis, and revealed that the responses to proteases were dependent on cerebral arteriole size, but testicular those showed little responses. The differences can be extrapolated to other transmitters. Here we examine whether there are any regional differences of arteriole responses to serotonin (5-HT), which plays a pivotal role in cerebral vasoconstriction and ischemic necrosis following subarachnoidal hemorrhage. [Methods]Arterioles (< 100 µm in diameter) were taken from brain and testis of rats. Isolated and cultured smooth muscles alter essential characteristics, therefore we used arteriole specimens which kept essential structural integrity. Specimens on the cover slips were perfused with Hepes-buffred Ringer solution containing 5-HT, analogues and antagonists. Spatio-temporal [Ca2+]i dynamics were analyzed by confocal microscopy (Nikon RCM/Ab). [Results] 5-HT induced smooth muscle contraction and increase of [Ca2+]i in small (<ca. 50 µm in diameter) and large (ca. 50-200 µm in diameter) arterioles of testis, and thapsigargin treatment inhibits the response. An agonist of 5-HT2 receptor (α-Met-5HT) induced strong responses, but responses to other agonists were faint. In cerebral blood vessels, large arterioles showed the responses, but not small those. After ether the withdrawing extracellular Ca2+ or thapsigargin treatments, the responses had not completely inhibited, indicating that both Ca2+ mobilization and influx were induced by 5-HT. Stimulation by various agonists indicated that cerebral large arteriole smooth muscles possessed 5HT1a, 1b, 2 (G-protein-coupled type) and 3 (ion channel type), and 5HT-1b and 2 play a main role in the 5-HT induced responses. Arterioles near the Willis's ring showed strong responses. [Conclusion] Regional differences of 5-HT receptors in cerebral blood vessels have to be consider in developing a new care to prevent fatal vasoconstriction of brain.

2105/B571

Paracrine Induction of Tks5 Adaptor Protein in Coronary Artery Smooth Muscle Cells by Breast Cancer Cells.

K. D. Barlow[1], W. Chen[2], C. McMahan[2], D. F. Seals[1], L. Metheny-Barlow[2,1]; [1]Cancer Biology, Wake Forest University Baptist Medical Center, Winston-Salem, NC, [2]Radiation Oncology, Wake Forest University Baptist Medical Center, Winston-Salem, NC

Physiological phenotypic modulation of mural cells (MC), including pericytes on capillaries and smooth muscle cells on larger vessels, is critical to vascular plasticity and responses to injury. However, MC plasticity is also susceptible to modulation by abnormal environmental cues, resulting in the promotion of pathological angiogenesis and vascular disease. We hypothesize that *in situ* carcinomas promote angiogenesis via paracrine signaling pathways that subvert MC function and induce an invasive phenotype. MC invasiveness may be promoted by altering the levels and/or functional status of key components of podosomes, dynamic, actin-rich adhesive structures that focally concentrate matrix remodeling proteases. Treatment of human coronary artery smooth muscle cells (CASMC) with medium conditioned by MDA-MB-231 breast cancer cells strongly induced accumulation of Tks5 protein, a Src tyrosine kinase substrate and adaptor protein previously shown to be required for the formation of podosomes in transformed fibroblasts and analogous structures in aggressive cancer cells. Induction of Tks5 protein was rapid and transient, occurring within 6 hrs of application and lasting no more than 24 hrs after withdrawal of conditioned medium (CM), and was partly dependent on transcription. Induction of Tks5 protein levels was partly dependent on activation of Src kinase, since (i) transient expression of activated Src induced Tks5 protein accumulation and (ii) pre-treatment with pharmacological inhibitors of Src attenuated induction of Tks5 protein by MDA-MB-231 CM. Moreover, inhibition of Src kinase also attenuated MAP kinase activation, while pharmacological inhibition of either MEK 1/2 or ERK2, as well as expression of an ERK2 dominant negative mutant, almost completely prevented Tks5 induction by CM. Together these experiments suggest that aggressive breast cancer cells may promote invasiveness in CASMC by inducing Tks5 adaptor protein via Src tyrosine kinase-dependent and -independent activation of ERK2 signaling. Ongoing experiments are determining whether the induction of Tks5 protein is sufficient to promote podosome formation and invasiveness in CASMC and alter functional interactions with endothelial cells.

2106/B572

A Novel Bio-Marker Exists in Atherosclerotic Lesions Can Be Detected by Unique Antibodies.

M. Mori[1], N. Yamaguchi[1], Y. Kamisaka[1], H. Kobayashi[1], A. Takano[1], H. Itabe[2], T. Takano[3], M. Fujimoto[1]; [1]Applied Pharmacology, Chiba Institute of Science, Choshi, Japan, [2]Biochemistry, Showa UNIV, Shinagawa, Japan, [3]Molecular Pathology, Teikyo Univ., Sagamiko, Japan
[Objective] We have examined to prepare unique monoclonal antibodies recognizing human and rabbit atherosclerotic aorta. Three different unique antigen and their property in lesion are investigated and discussed. **Phosphatidylcholine (PC)-free cholesterol (FC) complex** structures, a unique atheroma related antigen recognized by a novel monoclonal antibody **ASH1a/256C**. Truncated **Vitronectin**, which closely related with cell adhesion, also detected in human and WHHL rabbit atherosclerotic lesions by **EMR1b/244H**. **A unique PC structure** located in thickned intima can be detected by **LDR1a/104D**. **[Method]**. Antigens in lesion are detected by ELISA and immunoblotting. Their localization is also examined with fluorescent microscopy. Ruptured cell and foam cell death are detected by TUNEL and Annexin-V binding activity. FC-rich lipid droplets expressed in J774 cells are induced by WHHL rabbit serum **[Results & Discussion]** Using human or WHHL rabbit hyperlipidemic sera and J774 cells, we succeeded to express PC-FC complex on the surface of FC-rich lipid droplets as a foam cell death marker. PC-FC complex could be formed only when the cells were incubated with hyperlipidemic sera or AcLDL, not VLDL, LDL, moreover, VLDL enhances PC-FC complex formation by AcLDL; indeed, VLDL could not induce it by itself.Most of all lipid droplets located in atherosclerotic lesions, which contain high amount of FC. Vitronectin can localizes and associates in these FC-rich lipid droplets in the lesion. Most of all Vitronectins located here are reduced their molecular weight. Annexin-V positive foam cells were observed when the cells were incubated with WHHL hyperlipidemic sera, The cell incubated with U18666A, in order to block cellular cholesterol transport, could not be lead to apoptosis. Amount of FC and localization of FC in foam cells might be closely associated with foam cell death in atherosclerotic lesions. The UPR reaction marker, CHOP and XBP-1 were also detected in these dead cells. The hydrolysis and rearrangement of cellular cholesterol take place in foam cells to form the antigen; and lead to foam cell death. And one of the ECM protein, Vitronectin may be related with trap and keep FC-rich lipid droplet in thickned intima.

2107/B573

Does Acidosis Alter the Vascular Effect of Insulin through an Insulin-VEGF Cross-Talk Mechanism?

F. I. Achike[1], G. Subramaniam[2], M. Mustafa[2]; [1]Clinical Sciences, International Medical University, Kuala Lumpur, Malaysia, [2]Pharmacology, University of Malaya, Kuala Lumpur, Malaysia
Failure of insulin-induced vasorelaxation is a pathophysiologic feature of diabetes mellitus, a metabolic syndrome associated with ketoacidosis. We have shown that acidosis alters the effect of certain drugs. This study was designed to explore the mechanism of insulin vasodilatation at tissue and cellular levels and its possible alteration by acidosis. Thoracic aortic rings from Wistar-Kyoto (WKY) rats with (+ED) or without (-ED) endothelium were mounted in normal (pH 7.4) or acidotic (pH 7.2) Krebs solution for isometric tension recording. Phenylephrine (PE, 3.0 µM)-contracted tissues were exposed to insulin in the presence or absence of various inhibitors. Acidosis did not alter the concentration-dependent insulin-induced relaxation of +ED tissues. Endothelial denudation led to reduced effect of insulin under normal, but not acidotic pH. L-NAME, or Methylene Blue (MB) significantly reduced insulin responses in the +ED (but not the -ED) tissues. During acidosis, L-NAME did not affect insulin relaxation (+ED or -ED) but MB reduced it in +ED but not -ED tissues. Acidosis knocks off the EDNO-cGMP mediated insulin relaxation but compensates with an endothelium dependent, c-GMP mediated (non EDNO) mechanism which is perhaps VEGF coupled. We shall, therefore, explore insulin-VEGF cross-talk in vascular endothelial and smooth muscle cells under acidotic conditions.

Endothelial Cells (2108 – 2124A)

2108/B574

Hypoxia Induced Changes in the Contractile Machinery of Isolated Rat Pulmonary Microvascular Endothelial Cells (RPMVECs).

P. J. Bernal[1], S. C. Watkins[1], C. M. St Croix[1,2]; [1]Cell Biology and Physiology, University of Pittsburgh, Pittsburgh, PA, [2]Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA
We recently showed that alveolar hypoxia induced constriction of small non-muscularized intra-acinar arteries in isolated perfused mouse lungs, implicating a role for endothelium in modulating pulmonary vascular tone. We also reported that hypoxia-induced, NO-released zinc from the metal binding protein, metallothionein (MT), contributed to this response. At the cellular level, low oxygen tension was shown to induce an active contractile response in isolated rat pulmonary microvascular endothelial cells (RPMVECs). Experiments using the zinc-sensitive fluorophore, FluoZin-3 also showed that labile zinc was increased during hypoxic exposure in RPMVECs. In order to understand the structural changes underlying the hypoxia-induced contractile response, we examined actin-myosin dynamics as well as focal adhesion changes in response to low oxygen levels in collegen-embedded RPMVECs. Additionally, we investigated if increased intracellular levels of zinc could mimic the contractile response. Thrombin was used as known endothelial contraction agonist. RPMVECs were exposed to 30 minutes of either 2% oxygen, 10 uM zinc or thrombin. After fixation, the cells were imaged using confocal microscopy. We observed that contraction in response to either hypoxia, zinc or thrombin was accompanied by an increase in cell spanning actin stress fibers as well as increased actin-myosin interactions. An increase in cell attachment to the matrix was evidenced by increased recruitment of vinculin and paxillin to focal adhesion sites and zyxin to actin stress fibers during hypoxia, zinc and thrombin induced contraction. These processes were also analyzed in a time dependent manner using fluorescently tagged proteins. Live cell imaging corroborated the strengthening of actin stress fibers while total internal reflection imaging (TIRF) showed the recruitment of paxillin and vinculin to focal adhesions during hypoxia or zinc exposure. In conclusion, the cytoskeletal and focal adhesion changes observed during hypoxia and zinc exposure are associated with the force induction and cell stability necessary for an active contractile response. Funded by NIH HL 081421

2109/B575

Il-23 Activates Endothelial Cells Causing Intracellular Signalling and Spheroid Sprouting.

S. Dissing, T. Hammer, M. Hübschmann, K. Tritsaris; Department of Cellular and Molecular Medicine, Panum Institute, University of Copenhagen, Copenhagen, Denmark

Interleukin 23 (IL-23) stimulates survival and proliferation of Th17 cells that are implicated in autoimmune inflammatory diseases and IL-23 serves as an important cytokine regulator for these diseases. In addition, in psoriasis, IL-23 is overproduced by dendritic cells and keratinocytes and possibly drives development of psoriasis, a disease characterized by the formation of new, small capillaries. We studied to which extend IL-23 could activate endothelial cells from both blood and lymphatic vessels and initiate angiogenic and lymphangiogenic processes. RT-PCR on mRNA from human lymphatic endothelial cells revealed the presence of IL-12Rβ1 which is part of the IL-23 receptor complex. We found that activating lymphatic endothelial cells with IL-23 caused JAK2 phosphorylation as well as phosphorylation of Akt that was maximal after 30 minutes and a rapid, transient phosphorylation of mTOR that was maximal after 10 min. When lymphatic endothelial cells as well as porcine aorta endothelial cells were imbedded in collagen we found that IL-23 caused spheroid sprouting to an extent that resembled that observed with VEGF-C for lymphatic endothelial cells and VEGF-A and FGF for PAE cells. This process was further accelerated when IL-23 stimulated cells were incubated also in the presence of IL-20. IL-23 did not cause a rise in the intracellular [Ca2+] as seen with IL-20 (Tritsaris et al., PNAS, 104, 39 15364-69, 2007). In conclusion, in lymphatic endothelial cells IL-23 activates a receptor and intracellular signaling processes as well as spheroid cell sprouting. These data show that IL-23 might be implicated in processes that lead to formation of new lymphatic vessels and possibly also stimulates formation of blood capillaries.

2110/B576

Induction of Treg from PBMC by Interacting with Immunosuppressive Molecules on Oral Mesenchymal Stem Cells from Human Gingival Fibroblasts.

Y. Nagai, T. Kuroishi, A. Ohki, S. Sugawara; Oral Immunology, Tohoku University, Sendai, Japan

Despite high bacterial colonization, acute infections are rare in the oral mucosa. Recent study showed that mesenchymal stem cells show tolerogenic property. It is also known that periodontal tissues contain many kinds of mesenchymal stem cells such as the dental pulp stem cells, dental follicle cells and periodontal ligament stem cells. These reports lead us to the assumption that oral mesenchymal stem cells can contribute to the oral tolerogenic property. In this study, we attempted to establish mesenchymal stem cells from the human periodontal tissues, and investigated immunosuppressive function of the cells. A fibroblast-like cell line named H1C1 from extracted baby tooth periodontal tissue from 6 old of child was established by limiting dilution method and used for further research. The cell line had abilities to differentiate into osteocytes and chondrocytes and stably grew well at passage 15, therefore we termed H1C1 as the human mesenchymal stem cells. RT-PCR showed that H1C1 constantly expressed mRNA of co-stimulatory molecules B7-H1, B7-H2 and B7-H3, which are known as immunosuppressive molecules. Immunocytochemistry and FACS analysis showed H1C1 expressed B7-H3 protein. When human PBMC were co-cultured with H1C1, the population of CD4+CD25+Foxp3+cells was increased compared with monoculture of PBMC. These results suggest that human oral mesenchymal stem cells such as H1C1 have the abilities to suppress immunoreactions by co-stimulatory molecules and increasing regulatory T cells. In conclusion, we succeeded to establish the human oral mesenchymal stem cells named H1C1, which may contribute to the oral tolerogenic environment by cell-to-cell interactions and promoting the induction of regulatory T cells.

2111/B577

NFATc1 Regulates Lymphatic Endothelial Development.

R. Kulkarni[1,2], J. Greenberg[1,2], A. Akeson[1,2]; [1]Pulmonary Biology, Cincinnati Childrens Hospital Research Foundation, Cincinnati, OH, [2]Cancer and Cell Biology Graduate Program, University of Cincinnati, Cincinnati, OH

Murine lymphatic development starts at E10.5, with lymphatic endothelial cells (LEC) sprouting from the cardinal vein blood endothelial cells (BEC), a process requiring transcription factor Prox-1. However, the molecular mechanisms of lymphatic development are unknown. Human microvascular endothelial cells from lung (HMVEC-L) are heterogeneous population of BEC (high VEGFR2, low VEGFR3) and LEC (high VEGFR2, high VEGFR3). Microarray analysis comparing BEC and LEC identified transcription factors differentially expressed by LEC, including Prox-1 and NFATc1. NFATc1 is critical for cell lineage selection in cardiac valve morphogenesis and osteoclastogenesis. NFATc1 was colocalized with lymphatic markers Prox-1, VEGFR-3 and podoplanin on cardinal vein as LEC are specified and with these markers as LEC segregate into lymph sacs and matured embryonic as well as adult lymphatics, but not on blood vessels. In NFATc1 null mice, Prox-1, VEGFR-3 and podoplanin positive endothelial cells sprouted from the cardinal vein at E11.5, but failed to coalesce into lymph sacs. NFATc1 nuclear translocation and activation requires the phosphatase calcineurin. Embryos treated in utero with cyclosporine-A (calcineurin inhibitor) had cytoplasmic NFATc1 and diminished podoplanin expression on the lymphatics. In vivo, injury induced by lung-specific VEGF-A overexpression induces lymphangiogenesis and NFATc1 was expressed by the VEGF-A-induced lymphatics. Mice lacking the regulatory subunit of calcineurin, with diminished nuclear NFAT, failed to respond to VEGF-A with increased lymphangiogenesis. In vitro, VEGFR-3 and podoplanin expression by HMVEC-L was reduced by siRNA to NFATc1. This reduction was comparable to that seen with siRNA to Prox-1. In reporter assays, NFATc1 activated lymphatic-specific gene promoters showing an additive effect with Prox-1 on the FGFR-3 promoter. Co-immunoprecipitation and combinatorial siRNA analyses suggest NFATc1 and Prox-1 cooperation during injury-induced lymphangiogenesis. We propose that NFATc1 and Prox-1 are both required for lymphangiogenesis with the transcription factors showing an additive effect during development and synergism following injury-induced lymphangiogenesis.

2112/B578

Alternative RNA Splicing of Endothelin Receptor A and B in Various Cell Types of the Human Placenta.

M. Dieber-Rotheneder[1], U. Hiden[1], N. Ghaffari-Tabrizi[2], G. Desoye[1], M. Cervar-Zivkovic[1]; [1]Department of Obstetrics and Gynaecology, Medical University of Graz, Graz, Austria, [2]Institute of Pathophysiology and Immunology, Medical University of Graz, Graz, Austria

Objectives: In the human placenta endothelin-regulates vasotonus on endothelial and smooth muscle cells, whereas on the trophoblast it regulates cell proliferation, invasion and apoptosis. Its action is differently signaled through two endothelin receptor (ETR) subtypes, ETR-A and ETR-B. Several alternative splice variants of ETR were identified. Here we hypothesize that ETR splicing varies with gestational age and differs in trophoblast vs endothelial cells of the human term placenta. Methods: mRNA of placental tissue, primary cells [trophoblasts, arterial and venous placental endothelial cells (ECs)], and cell lines from first trimester and term were analyzed for full length ETR-A and ETR-B by Real time-PCR and known as well as unknown splice variants by sqRT-PCR. Primers spanning exons 2-5 or 2-3 of ETR-A and

against the ETR-B 8 exon- (8ex) and 7 exon- (7ex) transcript were used. The predominant ETR-A-DNA bands in agarose gel were excised, cloned and sequenced. Results: All tissues and cells expressed full length ETR-A and except of T trophoblasts a Δ3Δ4-deletion variant and a new ETR-A splice variant (partial Δ2 -Δ3- partial Δ2 deletion) with amplicon sizes of 551bp, 293 bp and 224 bp, respectively. Both ETR-B transcripts were found in all tissues and cell types. The 7ex transcript prevailed in placental tissue of first trimester (7ex:8ex-ratio: 2:1) and term (4:1) and in endothelial cells. The 8ex isoform was predominant in trophoblasts of first trimester (ratio: 1:2) and term (1:3). Arterious and venous EC did not differ. Conclusions: At term of pregnancy the endothelium is the predominant expression site of ETR-A whereas ETR-B prevails on trophoblasts. Gestational age does not alter ETR splicing. A new ETR-A splice variant was detected in tissue and ECs of the human placenta. ETR-B splicing differs between trophoblasts and ECs. Differential splicing may allow maternal and fetal endothelin-1 to induce different effects on the two major cell types of the placenta. (grant 11258, Jubilee Fund, OeNB, Vienna)

2113/B579

Elastin Fragments Activate Choroidal Endothelial Cells.

J. Skeie[1,2], J. Fishbaugh[3], R. Mullins[1]; [1]Carver Family Center for Macular Degeneration, University of Iowa, Iowa City, IA, [2]Biomedical Engineering, University of Iowa, Iowa City, IA, [3]Flow Cytometry Facility, University of Iowa, Iowa City, IA

Objective: Abnormal elastin metabolism has been observed in neovascular age-related macular degeneration. The presence of soluble elastin fragments in the choroid may be a stimulus for the pathologic activation of endothelial cells (ECs). We sought to analyze the effects of elastin fragments on choroidal EC migration and proliferation in vitro, as well as to evaluate potential elastin receptors. **Methods:** Migration of the chorioretinal EC line RF/6A and a primary culture of human choroidal ECs was measured using 8 µm diameter pore polycarbonate membranes. Migrating cells were exposed to elastin bioactive hexapeptides (BPs), elastin-derived peptides (EDPs), or balanced salt solution, and then counted using scanning electron microscopy. For proliferation assays, Rf/6a cells and human choroidal ECs were cultured in medium containing BPs, EDPs, kappa elastin, or balanced salt solution (control) for 72 hours. Cells were counted using a BD LSR flow cytometer. RNA from human choroidal ECs and human choroid was assayed for the presence of elastin receptor mRNA using RT-PCR. **Results:** Both cell types exposed to EDPs or BPs had increased migration compared to cells exposed to balanced salt solution alone ($p<0.05$). Endothelial cells exposed to EDPs, kappa elastin, or BPs, did not demonstrate a significant change in proliferation in comparison to controls. Human choroidal ECs and human choroid express mRNA transcripts encoding elastin binding proteins GLB1 (an alternatively spliced form of beta-galactosidase), and integrins alpha v and beta 3. **Conclusions:** Choroidal ECs express GLB1, alpha v integrin, and beta 3 integrin, which are elastin binding proteins that may elicit angiogenic responses from these cells in response to elastin fragments. In contrast to their effects on endothelial cell migration, elastin fragments do not appear to increase or decrease proliferation. Pathologic neovascular membrane formation in macular degeneration may therefore be due in part to elastin-mediated EC migration.

2114/B580

A Method to Study Endothelial Cell Mechanotransduction at the Apical Membrane.

E. Botvinick[1,2], J. Evora[1,2], S. Shreim[1,2]; [1]Beckman Laser Institute, University of California, Irvine, Irvine, CA, [2]Biomedical Engineering, University of California, Irvine, Irvine, CA

A microscope method has been developed to apply small-scale fluid flow fields to sub regions of the endothelial cell apical surface. These ultra-localized flow fields yield physiological magnitude stresses with low net force, thus minimizing signaling at the basolateral membrane where integrin signaling is known to occur. Spherical vaterite crystals are laser trapped and rotated by circularly polarized light near the cell surface. Measurement of crystal rotation rate and circular polarization as light exits the crystal allows calculation of applied torque and local apparent viscosity between the sphere and the cell. The fluid flow field is confined to the length scale of the crystal, a few microns, allowing ultra-fine viscosity measurements. The fluid environment near the cell surface can be probed as a crystal approaches the cell surface and begins to interact with the glycocalyx and with the plasma membrane. The rotating crystals can also be used to apply physiological-magnitude shear to local regions of the cell surface. Using apparent viscosity as an indicator, it is possible to position a rotating crystal as to selectively apply fluid shear to the glycocalyx. Finite element modeling indicates that the net force applied to the cell surface is orders of magnitude smaller than in a laminar flow chamber under equivalent shear stress. Results confirm low level Src kinase signaling at the basolateral membrane in response to rotating crystals as compared to signaling under laminar flow chamber conditions. In this way fluid shear mechanotransduction of the glycocalix can be measured independently from that of the basolateral membrane.

2115/B581

Moesin1 Is Required for VEGF-Dependent Endothelial Tube Formation In Vivo.

Y. Wang, J. J. Essner; Genetics, Development and Cell Biology, Iowa State University, Ames, IA

Endothelial tube formation is a critical step in the formation of functional blood vessels during angiogenesis, yet the molecular mechanisms that guide this process are relatively unknown. We show that zebrafish moesin1, a member of the ERM family of proteins involved in Actin-membrane associations, is required for endothelial tube formation in vivo. Knockdown of moesin1 during zebrafish development results in thin intersegmental vessels that are unable to transfer fluid. To better understand the function of Moesin1 and Actin in this process, we generated transgenic zebrafish which express Moesin1-egfp and Actin-mcherry fusion proteins in the endothelial cells. We find that Moesin1-egfp and Actin-mcherry proteins localize to the filopodia, cell junctions, and vesicles that form during endothelial tube formation. Importantly, the moesin1-egfp transgenic line is able to rescue the endothelial tube defects associated with morpholino knock down of Moesin1. This demonstrates that the Moesin1 knock down phenotype is specifically caused by disruption of Moesin1 activity and suggests that Moesin1 functions in a cell autonomous manner in endothelial cells during tube formation. Knock down of Moesin1 in the actin-mcherry transgenic line reveals that in the absence of Moesin1, fewer vesicles form in the endothelial cells. In addition, staining of moesin1 knock down embryos with antibodies against ZO-1 shows that in the absence of Moesin1, endothelial junctions fail to remodel during tube formation. These results suggest that lumen formation is closely coupled to cell junctional remodeling. Partial knock down of VEGF also prevented the accumulation of vesicles and subsequent lumen formation in the endothelial cells. In contrast, knock down of a Ser/Thr phosphatase, Ppp1ca, results in an increased number of large vesicles. These results are consistent with a model in which phosphorylation

levels regulate the activity of Moesin1 in endothelial cell lumen formation. Our results indicate that VEGF affects endothelial tube formation in vivo by regulating vesicle trafficking in endothelial cells and that Moesin1 plays a key role in this process.

2116/B582

Flow-Induced Changes in Lymphatic Endothelial Barrier Function.

J. W. Breslin, K. M. Kurtz; Physiology, School of Medicine, LSU Health Sciences Center, New Orleans, LA

The purpose of this study was to determine how lymphatic endothelial cells adapt and remodel in response to the various changes in shear stress encountered in the lymphatic vessel environment. We evaluated quantitative, real-time changes in lymphatic endothelial barrier function of cultured human dermal microlymphatic endothelial cells (HMLEC-d) by electric cell-substrate impedance sensing (ECIS), during no-flow and flow conditions. Cells were exposed to either continuous or pulsing (2 sec on, 2 sec off) flow for either 30 min. or 5 h, with periods of no-flow in between. Flow was applied at fluid shear stress values of 2.5, 5, 10, 20, or 38 dynes/cm^2. The results show that flow, either continuous or pulsatile, caused an increase in the transendothelial electrical resistance (TER), and indicator of barrier function. Turning flow off at 30 min. quickly reversed this response. When flow was allowed to continue for 5 h, TER initially increased, peaking near 15 min. after the onset of flow, and subsequently decreased to a steady-state that was 80-90% of baseline. When flow was stopped after 5 h, the TER decreased abruptly and then rose to a steady state level equivalent to baseline. The magnitudes of the responses were proportional to the shear stress applied. A mathematical model of current flow between and beneath cells based on our electrical impedance data shows that the flow-induced changes in TER were primarily due to altered cell-cell junctions. Blockade of PKA with 10 μM H-89, or ROCK with 5 μM Y-27632 did not inhibit the flow-induced response. These results suggest that lymphatic endothelial cells dynamically alter their morphology and barrier function in response to flow changes, however the precise mechanism remains elusive. Further studies will be required to determine how these responses influence lymph formation and propulsion in vivo.

2117/B583

DNA Binding Independent Regulation of the p21 gene by MEOX2 in endothelial cells.

J. M. Douville[1,2], D. Cheung[1,2], J. T. Wigle[1,2]; [1]Institute of Cardiovascular Sciences, St. Boniface General Hospital Research Centre, Winnipeg, MB, Canada, [2]Department of Biochemistry and Medical Genetics, University of Manitoba, Winnipeg, MB, Canada

The mesenchyme homeobox genes, MEOX1 and MEOX2, encode homeodomain transcription factor proteins. The homeodomain is an evolutionarily conserved DNA binding motif, however it has also been shown to mediate protein-protein interactions. MEOX2 is a key regulator of vascular cell function that controls cell proliferation via induction of the cyclin-dependent kinase inhibitor, p21. Study of Meox1/Meox2 double knockout mice has shown that the Meox proteins can partially compensate for one another during development. However, MEOX1 function in the vasculature has not yet been studied. The objectives of our study in primary human endothelial cells were to: 1) Establish the mechanism of MEOX2 transcriptional regulation of the p21 gene and 2) Determine whether MEOX1 is also a transcriptional activator of the p21 gene. In order to achieve these aims, MEOX proteins were expressed in primary human umbilical vein endothelial cells (HUVEC) via adenoviral transduction. The ability of MEOX proteins to regulate p21 expression was determined by luciferase reporter gene assays, western blot analysis and quantitative real-time PCR. Cellular proliferation was assessed by measuring bromodeoxyuridine (BrdU) incorporation. MEOX2 increased transcription from a p21 promoter 2-fold compared to an empty vector control and correspondingly increased endogenous p21 mRNA and protein levels when expressed in HUVECs. MEOX1 was also able to activate transcription from a p21 promoter and increase mRNA expression. Conversely, adenoviral transduction of MEOX1 did not significantly increase expression of the endogenous p21 protein in HUVECs. Interestingly, a DNA binding deficient, but not a homeodomain deleted, version of MEOX2 was able to induce endogenous p21 mRNA and protein expression in HUVECs. In all cases, increased p21 protein expression correlated with decreased BrdU staining. Taken together, we conclude that MEOX2 regulation of the p21 gene occurs via a mechanism that requires its homedomain, but is independent of DNA binding. Furthermore, although MEOX1 is able to activate transcription of p21, it is less potent than MEOX2 in its ability to activate p21 expression in endothelial cells.

2118/B584

VEGF Induces Rapid Upregulation of Intercellular Adhesion Molecule-1 (ICAM-1) on Human Lung Microvascular Endothelial Cells.

Q. Shen, E. S. Lee, R. L. Pitts, J. Tang, M. H. Wu, S. Y. Yuan; Department of Surgery, University of California Davis School of Medicine, Sacramento, CA

Dysregulation of endothelium barrier function in pulmonary microvasculature is crucial in many pathophysiological processes, such as diffuse inflammatory reactions and malignant metastasis, with the underlying mechanism still poorly understood. Vascular endothelial growth factor (VEGF) is a potential mediator of angiogenesis and microvascular hyperpermeability. To investigate the possible regulating effect of VEGF on endothelial ICAM-1, a critical molecule involved in both inflammation and tumor metastasis, human lung microvascular endothelial (HBMVEC-L) cells were treated with rhVEGF165 at elevating doses and various time courses. The expression levels of ICAM-1 on endothelial cells were then examined by SDS-PAGE/Western blot, flowcytometry, as well as immunocytochemistry. Further, blockage assays were conducted to investigate the potential role of VEGF receptor-2 (KDR) in VEGF-induced upregualtion of ICAM-1. Results show that VEGF increases lung microvascular endothelial ICAM-1 levels in a dose-dependent manner (plateau at 1nM after 2 hours of VEGF treatment). This VEGF-induced ICAM-1 expression is mediated through cell surface KDR. Further, following VEGF treatment at 1nM, a rapid surface upregulation of ICAM-1 was observed. These data strongly suggest a potential role of VEGF in neutrophil adhesion and subsequent infiltration in the process of inflammation.

2119/B585

VEGF-Induced Response Gene to Complement 32 (RGC32) Expression Inhibits VEGF-Related Angiogenesis in Hypoxic Endothelial Cells.

X. An[1,2], Y. Jin[1,2], J. Li[1]; [1]Cardiology, Beth Israel Deaconess Medical Center, Boston, MA, [2]Institute of Molecular Medicine, Peking University, Beijing, China

Objective: It has been suggested that hypoxia induces endothelial cells undergoing angiogenesis through HIF-1/VEGF pathway; however, hypoxia also leads cell to apoptosis. Here we provide that a novel hypoxia inducible gene, RGC32 (Response Gene to Complement 32), is involved in HIF-1/VEGF pathway. We describe the effect of VEGF on regulation of RGC32 and propose a potential activity of this effect. Methods and Results: Under hypoxic conditions (< 1% O2), RGC32 was induced up to 3.5 fold at 24 hours ($p<0.05$) in mRNA level in HUVECs, with an increase of RNA stability evidenced by the elongation of the half life from 1.96 hours in normoxia to 6.05 hours in hypoxia. In addition, RGC32 expression was induced by exposure of VEGF in the time- and dose- dependent manner, which was specific among various growth factors including FGF2, TGF-β, IL1-β, TNF-α, PDGF-BB treatment. Having incubated HUVECs with VEGF monoclonal antibody and VEGFR2 suppressor (SU4312, 0.6μM) before hypoxia, the hypoxia-induced RGC32 expression was diminished, suggesting that hypoxia induction of RGC32 is through VEGF activation. However, as a downstream gene of VEGF, RGC32 was not involved in the canonical VEGF pathway to stimulate angiogenesis. Instead, overexpression of RGC32 (o/e) in HUVECs resulted in the attenuation of endothelial cell proliferation (51%) and migration (45%) when compared to the controls. This result was additionally supported by siRNA of RGC32, in which the enhancement of proliferation and migration were observed. Furthermore, RGC32 o/e HUVECs showed a higher percentage of apoptotic cells analyzed by flow cytometry, which was confirmed by western blotting analysis with the increased cleaved caspase 3 and caspase 9. In Matrigel mouse model, RGC32 significantly inhibits VEGF-induced angiogenesis. Conclusion: This study revealed that RGC32 is a novel hypoxia inducible gene in endothelial cells. Hypoxia-induced RGC32 is via regulation of VEGF but inhibits angiogenesis and stimulates apoptosis in endothelial cells, which may provide negative feedback for the effect of VEGF. This highlights the important role that RGC32 plays in homeostasis in endothelial cells under hypoxia.

2120/B586

Induction of Apoptosis by Hypoxia Inducible Factor-1α-Mediated Upregulation of Regulator of G Protein Signaling 5 in Endothelial Cells.

Y. Jin[1,2], X. An[1,2], J. Li[1]; [1]Beth Israel Deaconess Medical Center, Boston, MA, [2]Institute of Molecular Medicine, Peking University, Beijing, China

Background: Gene regulation in response to hypoxia can lead endothelial cells in opposite directions: angiogenesis or apoptosis. G-protein coupling receptor (GPCR) signaling is involved in both processes. Regulator of G-protein signalling 5 (RGS5) is a regulator of G protein-mediated signalling that is strongly expressed in vessels during angiogenesis. On this basis we investigated the RGS5 in endothelial cells under hypoxic conditions. Methods and Results: The expressions of RGS5 mRNA and protein were immediately induced by hypoxia (1% oxygen) in 0.5 hours to 24 hours and peaked at 3 hours (2.3±0.25 fold) in HUVECs, however, the expression of RGS2 and RGS4 did not show any response to the hypoxic stimulus. Incubation of HUVEC in the presence of CoCl2 (150μM), DMOG (500μM) and DHB (200μM) resulted in an increase of the RGS5 mRNA. The impact of hypoxia on the activity of RGS5 promoter was only observed in HIF-1β +/+ cells, but not in HIF-1β -/- cells by using a luciferase assay. Overexpressing RGS5 (o/e) in HUVECs exhibited a reduction of growth rate without affecting the cell proliferation measured by 3H-Thymidine uptake. DNA ladder and Annexin V assay revealed that RGS5 enhanced HUVEC undergoing apoptosis, plus RGS5 o/e cells significantly increased activation of caspase-3 and the ratio of BAX/Bcl-2. Additionally, VEGF-mediated activation of P38 but not ERK1/2 was enhanced by RGS5 o/e HUVECs. Blockage of P38 activation by its inhibitor, SB203580 resulted in an attenuation of RGS5-enhanced apoptosis Conclusion: RGS5 is a novel HIF-1-dependent, hypoxia-induced gene, involved in the enhancement of endothelial apoptosis via VEGF-mediated MAPK signaling pathway. The impact of RGS5 on vascular function might reveal the involvement of GPCR signaling pathways in the hypoxia-mediated apoptosis of endothelial cells and provide a therapeutic potential in hypoxia/ischemia disorder.

2121/B587

The Role of β-Catenin in Microvascular Endothelial Cell Hyperpermeability.

B. Tharakan, T. L. Delmas, J. Hellman, F. A. Hunter, E. W. Childs; Surgery, Texas A&M Univ. Health Science Center COM and Scott and White Hospital, Temple, TX

β-Catenin has been shown to be an important regulator of the adherens junction complex. The activation of the intrinsic apoptotic signaling leads to caspase-3 mediated cleavage of β-catenin and subsequent disruption of adherens junction complex. The objective of our study was to determine the role of b-catenin in microvascular hyperpermeability utilizing rat lung microvascular endothelial cells (RLMEC). The cells were grown as monolayers in Transwell plates. The monolayers were transfected with pro-apoptotic peptide BAK (5 μg/ml). The change in permeability was determined by using FITC-albumin flux across the monolayer. To study the effect of caspase-3 on b-catenin at the endothelial cell adherens junctions, cells grown on chamber slides were transfected with active caspase-3 for 0-3 hours followed by immunofluorescence localization of β-catenin. The role of β-catenin in junctional integrity was studied by transfecting b-catenin siRNA duplexes to the monolayers. To understand the mechanism of β-catenin recycling and its relationship to the recovery from hyperpermeability, a time dependant caspase-3 transfection of RLMEC followed by co-localization of Myosin IIB and f-Actin was performed. BAK transfection significantly increased monolayer permeability ($p<0.05$). The increased permeability was associated with a loss of β-catenin at the adherens junctions. This was followed by the recovery of β-catenin at the junctions as well as recovery from hyperpermeability observed at 2 hr. β-catenin siRNA transfected cells demonstrated a substantial loss of β-catenin within the cell-to-cell junctions. The monolayers grown from the b-catenin siRNA transfected cells showed significant increase in permeability ($p<0.05$). There was an increased co-localization of Myosin IIB and f-Actin as the recovery process proceeded. Our results demonstrate that β-catenin is an integral component of the adherens junction regulating microvascular endothelial cell permeability. There is an increased formation of intracellular vesicles during the recovery stages of the monolayer suggesting β-catenin recycling during the recovery period.

2122/B588

Platelet Activating Factor Receptor-Mediated Activation of Endothelial Nitric-Oxide Synthase.

I. Knezevic[1], S. A. Predescu[1], C. Easington[1], R. Neamu[2], V. Brovkovych[3], D. N. Predescu[1]; [1]Pharmacology, RUMC, Chicago, IL, [2]Medicine, St. Raphael Hospital, New Haven, CT, [3]Pharmacology, University of Illinois, Chicago, IL

Regulated nitric oxide (NO) synthesis by the isoform III of nitric oxide synthase (eNOS) in endothelial cells (ECs) is essential for cardiovascular homeostasis. The expression and activity of eNOS is regulated by a range of transcriptional and posttranscriptional mechanisms including Ca2+/calmodulin binding, protein-protein interactions, phosphorylation, S-nitrosylation and probably subcellular localization. We addressed here, using morphological and biochemical studies as well as a real-time approach to measure NO levels, the mechanisms by which platelet activating factor (PAF) regulates the activity of eNOS. We have found that PAF receptor ligation induces a rapid (5 to 10 min) increase in NO production, followed by a longer (>30 min) period of reduction to basal NO levels. This PAF-induced burst of NO production was inhibited by 5 nM wortmannin and 10 nM PP2 pretreatment of cultured ECs monolayers. Moreover, studies to determine the phosphorylation status of serine (Ser), threonine (Thr) and tyrosine (Tyr) residues from eNOS molecule after PAF challenge indicated a dynamic phosphorylation of Ser1177 and Tyr residues, while Ser133 and Thr495 were dephosphorylated, by comparison to control ECs. Phosphorylation/dephosphorylation of Ser1177/Ser133 and Thr495 were inhibited by wortmannin, while c-Src inhibitors inhibited the phosphorylation of Tyr residue. Furthermore, the time-dependent phosphorylation/dephosphorylation events of eNOS correlated with NO production. Altogether, our findings indicate that PAF, via its receptor, modulates the phosphorylation of eNOS and thus, controls its enzymatic activity.

2123/B589

Regulation of Vascular Permeability Is Mediated through an Interaction of Filamin A and R-Ras in Human Coronary Artery Endothelial Cells.

G. S. Griffiths, M. Matter; Cardiovascular Research, John A Burns School of Medicine, Honolulu, HI

The cytoskeletal protein filamin A (FLNa) is required for cell-cell contact in vascular development and cardiac morphogenesis. FLNa null mice die of vascular defects including dilated vasculature, hemorrhage, and edema, suggesting abnormal vascular permeability. R-Ras, a small GTPase, is primarily expressed in endothelial cells and regulates vascular differentiation and blood vessel remodeling. FLNa is expressed in multiple cell types including endothelial cells. The objective of this investigation was to determine the functional mechanism of FLNa in endothelial cells. We examined whether FLNa and R-Ras interact and if altering their interaction via siRNA knockdown alters endothelial cell vascular permeability. A human coronary artery endothelial cell (HCEAC) model in which inflammation can be induced was used to investigate the role of FLNa and R-Ras. We examined the FLNa/R-Ras interaction by co-immunoprecipitation and co-localization by immunostaining. Functional significance was assessed using siRNA knock down of FLNa and R-Ras. Vascular permeability was measured by an Electrical Cell Impedance Sensing device. In HCAECs, FLNa and R-Rras co-immunoprecipitate and co-localize at the plasma membrane. Upon inflammation, FLNa expression increased when compared to non-inflamed cells while R-Ras expression decreased. Endothelial cell vascular permeability increased in cells that had FLNa, R-Ras or FLNa/R-Ras knocked down compared to normal HCAECs. TNFα treatment resulted in increased vascular permeability in cells with R-Ras or R-Ras/FLNa knocked down compared to TNFα treated controls. TNFα treatment induced a decrease in endothelial cell vascular permeability in cells expressing reduced FLNa compared to TNFα treated controls. A novel interaction between FLNa and R-Ras is demonstrated, suggesting that the R-Ras/FLNa association promotes regulation of endothelial cell permeability.

2124/B590

Forskolin Increases Angiogenesis through the Coordinated Cross-Talk of PKA-Dependent VEGF Expression and Epac-Mediated PI3K/Akt/eNOS Signaling.

Y. Kim[1], C. Kim[1], H. Lee[2]; [1]School of Medicine, Kangwon National University, Chunchon, South Korea, [2]Division of Life Sciences, Kangwon National University, Chunchon, South Korea

Forskolin, a potent activator of adenylyl cyclases, elevates intracellular cAMP levels. This compound has been implicated in modulating angiogenesis, but the underlying mechanism has not been clearly elucidated. In this study, we investigated the molecular signal mechanism by which forskolin regulates angiogenesis. Forskolin stimulated proliferation, migration, and tube formation in HUVECs in vitro and angiogenesis in vivo, which was accompanied by phosphorylation of CREB, ERK, Akt, and endothelial nitric oxide synthase (eNOS) as well as nitric oxide (NO) production and VEGF expression. Forskolin-induced CREB phosphorylation, VEGF promoter activity and VEGF expression were blocked by the PKA inhibitor PKI. Moreover, phosphorylation of ERK by forskolin was inhibited by the MEK inhibitor PD98059, but not PKI. The forskolin-induced Akt/eNOS/NO pathway was completely inhibited by the phosphatidylinositol 3-kinase (PI3K) inhibitor LY294002, but not significantly suppressed by PKI. These inhibitors and a NOS inhibitor partially inhibited forskolin-induced angiogenesis. Furthermore, the Epac activator 8-(p-Chlorophenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate, an exchange protein directly activated by cAMP, promoted the Akt/eNOS/NO pathway and ERK phosphorylation, but did not induce CREB phosphorylation and VEGF expression. The angiogenic effect of the Epac activator was diminished by the inhibition of PI3K and MEK, but not by the PKA inhibitor. These results suggest that forskolin stimulates angiogenesis through coordinated cross-talk between two distinct pathways, PKA-dependent VEGF expression and Epac-dependent ERK activation and PI3K/Akt/eNOS/NO signaling.

2124A/B591

Rho Kinase Activity Determines Baseline Cell-Generated Traction Stresses in Confluent Endothelial Monolayers.

R. Krishnan[2], C. M. Beckers[1], V. W. Van Hinsbergh[1], J. J. Fredberg[2], G. P. van Nieuw Amerongen[1]; [1]Physiology, VUmc University Medical Center, Amsterdam, Netherlands, [2]Department of Environmental Health, Harvard Public School of Health, Boston, MA

Acto-myosin generated tension has been known for a long time to be of vital importance for regulation of endothelial barrier function. This mechanical tension is balanced by traction stresses exerted by the endothelial cells (ECs) at cell-cell and cell-substrate interactions. Despite its biological significance, traction distribution within an intact monolayer has never been determined. Here, we describe a novel technique to

measure cell-substrate tractions in EC monolayers and demonstrate its utility by quantifying the effects of the potent hyperpermeability-inducer thrombin. ECs were cultured within 150 µm diameter circular collagen islands micropatterned upon a deformable polyacrylamide gel substrate with embedded fluorescent nano-beads. Cell exerted substrate deformations were determined by comparing images of these nano-beads obtained during the experiment with a reference image obtained subsequent to detaching the ECs. Cell tractions were computed from the displacement field using constrained Fourier transform traction microscopy (FTTM). We observed that considerable tractions were exerted by the monolayer under baseline conditions (average RMS traction = 121±76 Pa, n=4). Remarkably, baseline traction forces were not randomly distributed but were arranged in irregular hotspots. Importantly, these traction hotspots coincided with sites where of gaps formed between endothelial cells upon stimulation with thrombin. Thrombin induced a robust 3-fold increase in contractile moment. Preincubation for 30 minutes with 10 mM Y-27632 reduced baseline traction by 50 ± x % (n=4; $p<0.05$), but did not prevent thrombin-induced contractility. In summary, we describe here a novel technique for quantitative mapping of traction stresses in EC cell monolayers. These data reveal that traction distribution in an EC monolayer is remarkably non-uniform at baseline with hotspots representing sites susceptible to gap formation. Furthermore, they show that Rho kinase is an essential regulator of base-line tractions, rather than mediating agonist-induced endothelial hyper-contractility. Financial support was received from The Netherlands Heart Foundation (NHS2003-T032).

Synapse Formation and Function (2125 – 2138)

2125/B592

Possible Role in Neurotransmitter Release and Signaling of a Multi-Domain Adaptor Protein, p140Cap, at the Presynapse.

K. Nagata[1], H. Ito[1], K. Atsuzawa[2], K. Sudo[1], P. Di Stefano[3], I. Iwamoto[1], R. Morishita[1], P. Defilippi[3], S. Takei[4], R. Semba[4], T. Asano[1], N. Usuda[2]; [1]Molecular Neurobiology, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Japan, [2]Anatomy, Fujita Health University School of Medicine, Toyoake, Japan, [3]Molecular Biotechnology Center, University of Turino, Turin, Italy, [4]Pathology, Institute for Developmental Research, Aichi Human Service Center, Kasugai, Japan

p140Cap is an adaptor protein considered to play pivotal roles in cell adhesion, growth and Src tyrosine kinase-related signaling in non-neuronal cells. It is also reported to interact with a presynaptic membrane protein, SNAP-25, and may participate in neuronal secretion. However, properties and precise functions of p140Cap in neuronal cells are almost unknown. We thus carried out comprehensive analyses to elucidate functions of p140Cap in neuronal tissues. We show, using biochemical analyses, that p140Cap is expressed in rat brain, in a developmental stage-dependent manner, and is relatively abundant in the synaptic plasma membrane fraction in adults. Immunohistochemistry showed localization of p140Cap in the neuropil in rat brain and immunofluorescent analyses detected p140Cap at synapses of primary cultured rat hippocampal neurons. Electron microscopy further revealed localization at presynapses along plasma membranes. Screening of p140Cap-binding proteins identified a multi-domain adaptor protein, vinexin, whose third SH3 domain interacts with the C-terminal Pro-rich motif of p140Cap. Immunocomplexes between the two proteins were confirmed in COS7 and rat brain. p140Cap appeared to affect intracelluar localization of vinexin and interaction of vinexin with its binding partners such as N-WASP. We also clarified that a presynaptic protein, synaptophysin, interacts with p140Cap. These results strongly suggest that p140Cap is involved in neurotransmitter release, and synapse formation/maintenance and signaling.

2126/B593

Docking of Axonal Mitochondria by Syntaphilin Controls Their Mobility and Affects Short-Term Synaptic Facilitation.

J. Kang, P. Pan, J. Tian, Z. Sheng; Synaptic Function Section, NINDS, NIH, Bethesda, MD

Proper distribution of mitochondria within axons and at synapses is critical for neuronal function. While one-third of axonal mitochondria are mobile, a large proportion remains in a stationary phase. However, the mechanisms controlling mitochondrial docking within axons and at synapses remain elusive. Here we report a role for axon-targeted syntaphilin (SNPH) in mitochondrial docking through its interaction with microtubules. Axonal mitochondria that contain exogenously or endogenously expressed SNPH lose mobility. Deletion of the mouse snph gene results in a substantially higher proportion of axonal mitochondria in the mobile state and reduces the density of mitochondria in axons. The snph mutant neurons exhibit enhanced short-term facilitation during prolonged stimulation by affecting calcium signaling at presynaptic boutons. This phenotype is fully rescued by reintroducing the snph gene into the mutant neurons. Thus, our study reveals that SNPH acts as a receptor for docking/retaining mitochondria through an interaction with the microtubule-based cytoskeleton. Such a mechanism may enable neurons to maintain proper densities of stationary mitochondria within axons and in the proximity of synapses. Furthermore, our findings provide cellular and genetic evidence that the mobility and density of inter-bouton mitochondria play a critical role in short-term facilitation during high frequency stimulation. (The study was recently published: Kang et al., Cell 2008)(Supported by the Intramural Program of NINDS, NIH).

2127/B594

KIF1Bbeta/KIF1A-Mediated Axonal Transport of Presynaptic Regulator Rab3.

S. Niwa, Y. Tanaka, N. Hirokawa; Department of Cell Biology, School of Medicine, University of Tokyo, Tokyo, Japan

Synaptic proteins are synthesized in the cell body and transported down the axon by microtubule dependent motors. We previously reported that KIF1Bbeta/KIF1A motors are essential for transporting synaptic vesicle precursors; however the mechanisms that regulate this process, such as cargo recognition and control of cargo loading and unloading remain largely unknown. Here, we show that DENN/MADD is a critical part of the regulation mechanism, through direct interaction with the stalk domain of KIF1Bbeta/KIF1A. We also show that DENN/MADD preferentially binds to GTP-Rab3 and acts as a new Rab3 effector. These molecular interactions are considered fundamental because sequential genetic perturbations revealed that KIF1Bbeta/KIF1A is essential for the transport of DENN/MADD and Rab3, while DENN/MADD is essential for the transport of Rab3. GTP-Rab3 was more effectively transported than GDP-Rab3, suggesting that the nucleotide state of Rab3 regulates axonal transport of Rab3-carrying vesicles through preferential interaction with DENN/MADD.

2128/B595

The PAR-6 Polarity Protein Regulates Dendritic Spine Morphogenesis through P190 Rhogap and the Rho Gtpase.

H. Zhang, I. Macara; Center for Cell Signaling, University of Virginia, Charlottesville, VA

The majority of excitatory synaptic transmission in the brain occurs at dendritic spines, which are actin-rich protrusions on the dendrites. The asymmetric nature of these structures suggests that proteins regulating cell polarity might be involved in their formation. Indeed, the polarity protein PAR-3 is required for normal dendritic spine morphogenesis. However, this function is independent of association with atypical protein kinase C (aPKC) and PAR-6. Here we show that PAR-6 together with aPKC plays a distinct but essential role in spine morphogenesis. Knockdown of PAR-6 inhibits spine morphogenesis, whereas overexpression of PAR-6 increases spine density, and these effects are mediated by aPKC. Using a fluorescence resonance energy transfer (FRET) biosensor, we further show that p190 RhoGAP and RhoA act downstream of the PAR-6/aPKC complex. These results define a role for PAR-6 and aPKC in dendritic spine biogenesis and maintenance, and reveal an unexpected link between the PAR-6/aPKC complex and RhoA activity. Ongoing studies are aimed at elucidating the mechanism by which the PAR-6/aPKC complex regulates p190 RhoGAP activity.

2129/B596

Sonic Hedgehog Regulates Dendritic Spine Formation through a Non-canonical Pathway.

N. Sasaki, M. Kengaku; Neural Cell Polarity, RIKEN Brain Science Institute, Wako, Japan

During development of the vertebrate central nervous system (CNS), Sonic hedgehog signaling pathway plays essential roles in various processes including dorsoventral patterning of the neural tube, proliferation of neural progenitors and guidance of growing axons. Alterations in sonic hedgehog signaling pathway cause brain tumors such as medulloblastoma. Here, we found that sonic hedgehog signaling might be involved in dendritic spine formation in CNS neurons. In-situ hybridization revealed expression of sonic hedgehog and its receptors Patched1 and Smoothened in cerebellar Purkinje neurons and hippocampal pyramidal neurons during early postnatal development of mice. Treatment with sonic hedgehog enhanced spine maturation in cultured hippocampal neurons. Conversely, inhibition of Smoothened activity by a specific inhibitor cyclopamine retarded spine maturation, resulting in the increment of immature dendritic filopodia. GST-pull down assays revealed that Smoothened bound to key moleules that regulates spine formation via the cytoplasmic C-terminal region. Inhibition or activation of Gli-dependent transcriptional pathway did not affect spine morphology, suggesting that sonic hedgehog might regulate dendritic spine formation in CNS neurons through a non-canonical pathway downstream of Smoothened.

2130/B597

The Reduction of Ubiquitin Pool Caused by the Loss of Usp14 Results in Aberrant Synaptic Growth and Maturation at the Neuromuscular Junctions.

P. C. Chen[1], L. Qin[2], X. Li[2], B. J. Walters[1], L. Mei[2], S. M. Wilson[1]; [1]Department of Neurobiology, U. Alabama at Birmingham, Birmingham, AL, [2]Institute of Molecular Medicine and Genetics, Department of Neurology, Medical College of Georgia, Augusta, GA

Recent research highlights that the synaptic defect plays an important role in the progress of neurodegenerative disorders. The ubiquitin proteasome system (UPS) has been shown to contribute to the pathogenesis of neurodegenerative diseases and regulates synapse development. However, how the impairment of UPS disrupts synaptic development in the mammalian nervous system has not been well characterized. Previous studies showed that Usp14, a proteasome associated deubiquitinating enzyme recycles the ubiquitin and maintains the ubiquitin pool. The loss-of-function mutation of *Usp14* in the *ataxia* (ax^J) mice results in reduced monomeric ubiquitin levels, failure of synapse function, and progressive neurological disorders such as tremor, muscle wasting and paralysis. In this report, we use ax^J mice and several transgenic lines to study the mechanism by which loss of Usp14 results in synaptic defects at the neuromuscular junctions (NMJs). In the ax^J mice, progressive pre-synaptic morphological defects at NMJ were detected from P7, including neurofilament accumulation, nerve terminal swelling and sprouting. In addition, the majority of the post-synaptic endplates display immature morphology with less arborization by P14~P21. Transgenic restoration of Usp14 in neurons by Thy1-Usp14 transgene corrected the monomeric ubiquitin levels in motor neuron circuit, muscle wasting, and NMJ deficits of the ax^J mice. Surprisingly, restoration of neuronal ubiquitin levels by Thy1-Ubiquitin transgene can partially rescue the neuromuscular phenotypes and NMJ defects, in which more than 50% of NMJs were corrected from nerve terminal sprouting and the impairment of endplate maturation. Taken together, these results suggest that NMJ synapse maturation and maintenance requires Usp14 on the presynaptic side to maintain the ubiquitin pool. Our findings implicate ubiquitin levels as key indicators of neuronal health and suggest that drug therapies targeted to this pathway may provide benefits to certain neurodegenerative diseases.

2131/B598

Point Mutation Analysis of Alpha-Dystrobrevin's Individual Tyrosine Residues.

J. Gingras[1], R. M. Grady[2], J. R. Sanes[1]; [1]Molecular & Cellular Biology, Harvard University, Cambridge, MA, [2]Pediatrics, Washington University School of Medicine, St Louis, MO

The dystrophin-glycoprotein complex (DGC) plays a crucial role in linking the cytoskeleton to the extracellular matrix in skeletal muscle. Mutation of several DGC components lead to muscular dystrophy and/or aberrant maturation of the neuromuscular junction (NMJ). Genetic studies showed that alpha-dystrobrevin (αDB) is a critical component of the synaptic DGC: targeted αDB mutants show mild dystrophy but dramatic defects in postsynaptic maturation. (Grady et al., Nature Cell Biol, 1999, Neuron, 2000). One alternatively spliced form of αDB, αDB1, is concentrated at the NMJ; this isoform can undergo tyrosine phosphorylation at its unique C-terminus, and only tyrosine phosphorylatable aDB1 can rescue synaptic defects in αDB1 mutant mice (Grady et al., JCB, 2003). We generated phospho-specific antibodies to two of three tyrosine-phosphorylatable sites in αDB1, and used them to show that αDB1 phosphorylation is dynamically regulated during postnatal development and by innervation. To understand how aDB1 functions, we generated GFP-αDB1 constructs in which one, two or all of three tyrosine phosphorylation sites (Y698, Y706 and Y723) were mutated. The constructs were introduced into cultured muscle cells, and their effects on targeting of αDB1 to acetylcholine receptor (AChR) clusters, as well as their effect on clustering, was assessed. Targeting of αDB1 was independent of phosphorylation, but overexpression of the constructs disrupted AChR cluster morphology. Disruption was greatest in triple Y-F mutants, significant in double mutants and undetectable in single mutants or wild-type

αDB1. Mutations of Y706 or Y723 had more striking effect than mutation of Y698. Together, our results suggest that αDB1 tyrosine phosphorylation is not essential for AChR clustering, but necessary for proper maturation of the clusters. (This work was supported by NIH and by a FRSQ fellowship to J.G.)

2132/B599

Biochemical and Histological Analyses of Dysbindin-1 in Rat Brain.

H. Ito, R. Morishita, K. Sudo, I. Iwamoto, T. Shinoda, K. Okamoto, K. Nagata; Dept. of Mol. Neurobiol., Aichi Human Service Center, Kasugai, AIchi, Japan

Genetic variations in dysbindin-1 (dystrobrevin-binding protein-1, DTNBP1) are one of the most commonly reported variations associated with schizophrenia. Schizophrenia is now considered as a neurodevelopmental disorder resulting in abnormalities of synaptic connectivity. However, the exact role of dysbindin-1 in the neuronal development is yet obscure. In this study, we aimed to clarify the physiological function of dysbindin-1 in neuronal tissues. We developed a polyclonal antibody for dysbindin-1 and confirmed the specificity by Western blot analysis. Using this antibody, we analyzed the expression profiles of dysbindin-1 in various rat tissues and found that the isoform with molecular mass of about 50 kDa was clearly detected in several tissues including brain, lung, adrenal gland, uterus, ovary and testis. In contrast, the isoform with molecular mass of about 40 kDa was only detected in brain. We also examined the developmental change of the expression of dysbindin-1 in rat brain and found that the level of 50 kDa isoform decreased gradually during the embryonic to postnatal development while the level of 40 kDa isoform increased at postnatal developmental stage. Immunohistochemical analysis of rat brain revealed the distribution of dysbindin-1 at soma and dendrites of cortical neurons, hippocampal neurons and cerebellar Purkinje cells. From immunofluorecent analysis, we found the localization of dysbindin-1 at spine-like structure of primary cultured rat hippocampal neurons. To further explore the physiological roles of dysbindin-1 in neuronal development, we are now seeking the binding partner for dysbindin-1 using yeast two-hybrid screening.

2133/B600

Depletion of Adult Hippocampal Neurogenesis Does Not Block the Formation and Extinction of Contextual Fear Memory.

D. Jang, H. Ko, J. Son, B. Kaang; Biological Science, Seoul National University, Seoul, South Korea

Newborn neurons in the subgranular zone (SGZ) of the hippocampus incorporate into the dentate gyrus (DG) and become maturated. Numerous studies have focused on the hippocampal neurogenesis for its importance in learning and memory. However, it is largely unknown whether hippocampal neurogenesis is involved in memory extinction per se. We sought to examine the possibility that hippocampal neurogenesis may play a critical role in the extinction of hippocampus-dependent contextual fear memory. Using methylazoxymethanol acetate (MAM) and X-ray irradiation, hippocampal neurogenesis was impaired in adult mice. In our experimental condition, impaired hippocampal neurogenesis did not affect not only on the extinction but also on the formation of contextual fear memory. These results suggest that hippocampal neurogenesis is not involved in the extinction or erasure of hippocampus-dependent contextual fear memory.

2134/B601

Metabolic Modulation of Synaptic Connections in the Mouse Retina.

R. Barlow[1], N. Cuenca@ua.es[2], Y. Umino[1], L. Fernandez-Sanchez[2], K. Cusato[1], D. Everhart[1], E. Solessio[1]; [1]SUNY Upstate Medical School, Syracuse, NY, [2]University of Alicante, Alicante, Spain

Metabolic stress in the form hypoglycemia leads to late-onset retinal degeneration in transgenic mice. We found synaptic remodeling, decrease in retinal function, and loss of vision in mice rendered chronically hypoglycemic from a null mutation of the glucagon receptor gene, Gcgr. We assessed retinal function with the ERG and retinal anatomy with single and double immunostaining using antibodies to gamma-transducin, recoverin, bassoon, kinase-C, calbindin, and synaptophysin. We compared retinal morphology of Gcgr-/- mice with reduced vision to that of Gcgr+/- mice with normal vision. Gcgr-/- mice have near normal cone photoreceptor population but reduced rod population. Bassoon and Kinase-C antibodies show a reduction of synaptic terminals in the OPL with dendritic processes of rod bipolar cells disconnecting with photoreceptor terminals. Also calbindin staining shows a retraction of horizontal cell terminals. These morphological changes correlate with a >50% thinning of the outer plexiform layer. Horizontal and bipolar cells processes sprout into the ONL in areas near the optic nerve. These morphological changes in the OPL of hypoglycemic mice correlate with a reduced ERG b-wave and loss of retinal function. Diet-induced euglycemia can partially rescue age-related loss of vision and synaptic connectivity in hypoglycemic Gcgr-/- mice. This is the first known instance of a loss of synaptic connectivity in the retina without associated cell death. Linkage between synaptic remodeling and hypoglycemia in mice may highlight the importance of glycemic control in diabetes and retinal diseases related to metabolic stress as macular degeneration. (Supported by grants BFU2006-00957/BFI, ONCE and Fundaluce to NC, NIH-R01EY00667 to RB and by RPB, and Lions of Central New York.)

2135/B602

Physiological Role of Epilepsy-Related Ligand LGI1 in Synaptic Function.

Y. Fukata[1,2], A. Watanabe[3], T. Iwanaga[1], M. Fukata[1,2]; [1]Division of Membrane Physiology, Department of Cell Physiology, National Institute for Physiological Sciences, Okazaki, Japan, [2]PRESTO, Japan Science and Technology Agency, Chiyoda, Japan, [3]Department of Vascular Dementia Research, National Center for Geriatrics and Gerontology, Obu, Japan

Finely tuned synaptic transmission in the brain provides the molecular basis for learning and memory. The misregulation of synaptic transmission is involved in the pathogenesis of various neurological disorders such as epilepsy. LGI1 is a neuronal secreted protein and its mutations cause autosomal-dominant partial epilepsy with auditory features. We recently found that LGI1 is a ligand for ADAM22 and enhances AMPA type glutamate receptor-mediated synaptic transmission. Here, we generated knockout mice for LGI1 and found that all homozygous mutant mice display severe seizures and die within postnatal three weeks. To explore the mode of action of LGI1 ligand, we purified LGI1 containing complex by taking advantage of a transgenic mouse expressing an epitope-tagged LGI1. We identified ADAM22 and a closely-related ADAM23 as major LGI1 receptors in brain. Interestingly, the epileptic phenotype of LGI1 null mouse was very similar

to the reported neurological phenotype of ADAM22 and ADAM23 knockout mice. ADAM22 and ADAM23 were completely missing from synaptic fraction in LGI1 knockout mice. We will discuss how LGI1/ADAM22/ADAM23 regulates synaptic function and how disorganization of this protein complex causes epilepsy.

2136/B603

Asymmetric PIP3 Signals Mediate Nerve Growth Cone Chemoattraction.

S. Henle[1], E. Liang[1], G. Wang[2], M. Wu[3], M. Poo[3], J. Henley[1]; [1]Neurosurgery, Mayo Clinic, Rochester, MN, [2]Molecular and Cell Physiology, Stanford University, Stanford, CA, [3]Molecular and Cell Biology, UC Berkeley, Berkeley, CA

Gradients of attractive and repulsive chemotropic factors guide the pathfinding of axons in the developing nervous system. The cellular action of many guidance factors is mediated by cytoplasmic Ca^{2+} signals, but the link between surface receptor activation and Ca^{2+} signaling is largely unknown. In migrating leukocytes and amoeboid cells, polarized elevation of the plasma membrane lipid PtdIns(3,4,5)P3 (PIP3) is known to mediate the detection of a chemoattractant gradient and initiates directed chemotaxis. Here we report that a gradient of an axon guidance factor, brain-derived neurotrophic factor (BDNF), also rapidly induces asymmetric PIP3 accumulation in the nerve growth cone, as revealed by translocation of the fusion protein Akt-PH-GFP to the plasma membrane. The activation of both phosphatidylinositol-3 kinase (PI3K) and Akt was necessary for BDNF and netrin-1 induced chemoattraction, and asymmetric application of exogenous PIP3 was sufficient to induce growth cone turning. Furthermore, focal application of exogenous PIP3 rapidly activated TRPC (transient receptor potential C) channels in the growth cone membrane, leading to Ca^{2+} influx, a process dependent upon the activities of PI3K and Akt. Our findings support the notion that asymmetric PIP3 signals at the growth cone membrane may serve to link focal receptor activation with TRP channel activity and Ca^{2+} signaling during chemoattractive axon guidance.

2137/B604

Myelin-Associated Glycoprotein Induced Modulation of Beta-1 Integrin during the Guidance of Nerve Growth Cones.

J. Hines[1], J. Henley[2]; [1]Mayo Graduate School, College of Medicine, Mayo Clinic, Rochester, MN, [2]Departments of Neurologic Surgery, Physiology & BME, Mayo Clinic, Rochester, MN

Myelin-associated glycoprotein (MAG) is a potent inhibitor of axon regeneration and an important therapeutic target following spinal cord injury. Additionally, MAG can act as an axon guidance cue by repelling nerve growth cones, which are the motile tips of growing axons. We hypothesized that MAG regulates the internalization of surface receptors and adhesion complexes to inhibit axon outgrowth and induce growth cone chemorepulsion. Here we report that MAG stimulates endocytosis of the adhesion receptor β1-integrin in growth cones migrating on fibronectin. This internalization required PI-3 kinase and Ca++ signaling, but not Rho kinase (ROCK), a known signaling component downstream of MAG. Using morpholino knockdown, we show that MAG-induced inhibition of axon outgrowth is mediated by β1-integrin. To address whether MAG-induced endocytosis participates in growth cone turning, we developed a novel FM-dye assay to monitor endocytosis in growth cones migrating through a microscopic gradient of MAG. Using rapid confocal microscopy, we find elevated endocytosis on the side of the growth cone nearest the MAG gradient. Together, these data support the notion that MAG-induced integrin modulation is a novel mechanism for inhibiting axon outgrowth and facilitating growth cone repulsion by asymmetrically altering integrin-mediated adhesion.

2138/B605

rAAV8-Mediated Protein Anchoring of Asymmetric Acetylcholinesterase (Ache) to the Synaptic Basal Lamina at the Neuromuscular Junction (NMJ).

M. Ito[1], Y. Suzuki[1], T. Okada[2], T. Fukudome[3], T. Yoshimura[4], A. Masuda[1], S. Takeda[2], E. Krejci[5], K. Ohno[1]; [1]Div of Neurogenetics, Ctr for Neurological Diseases and Cancer, Nagoya Univ. Grad. Sch. of Med, Nagoya, Japan, [2]Dept of Molecular Therapy, NCNP, Tokyo, Japan, [3]Dept of Neurology, Nagasaki Med Ctr of Neurology, Nagasaki, Japan, [4]Dept of Occupational Therapy, Nagasaki Univ, Nagasaki, Japan, [5]INSERM U686, Paris, France

Congenital endplate AChE deficiency is caused by mutations in *COLQ* encoding collagen Q (ColQ). ColQ makes a triple helical structure and anchors the AChE catalytic subunit to the synaptic basal lamina in the form of asymmetric AChE. There has been no rational therapy for this disorder. Here we exploited AAV8 (adeno-assosiated virus serotype 8), which has tropism for skeletal muscle, carrying the human *COLQ* gene (rAAV8-*COLQ*) to anchor asymmetric AChE to the synaptic basal lamina of *Colq*-knockout mice. We injected ~2 x 10^{12} vector genomes into the tail vein of *Colq*-knockout mice at postnatal weeks 3 to 4. In three weeks after injection of 2 x 10^{12} vector genomes, motor symptoms recovered to the level of the wild-type littermates, which lasted at lest 8 months after injection without a decline. Cytochemical and immunohistochemical analyses disclosed high AChE activity and ColQ expression at the NMJs. Sedimentation analysis demonstrated both globular and asymmetric forms of AChE in skeletal muscles, which were indistinguishable from those of wild-type littermates. Miniature endplate potential (MEPP) recordings revealed shortened decay time constants and reduced MEPP amplitudes. A decrement of compound muscle action potentials (CMAPs) in response to 2-Hz nerve stimulations was also improved. On electron micrographs, treated mice exhibited normal NMJs, but some NMJs were similar to those of untreated mice. In order to examine if the excreted asymmetric AChE was indeed delivered to remote muscle fibers, we intramuscularly administered AAV8 to the hindlimbs. We observed an expression of asymmetric AChE at the NMJs of the forelimbs, which readily explained why the motor functions of our intravenously treated mice were restored almost to the normal level, even though *COLQ* was introduced into a limited number of skeletal muscle fibers. Our current studies imply feasibility of the "protein anchoring therapy" in which a recombinant extracellular matrix molecule expressed in a limited number of cells can be delivered to the target organ throughout the whole body by exploiting its tissue-targeting signal of the recombinant molecule itself.

Development and Aging (2139 – 2158)

2139/B607

Topology Rather Than Cell Lineage Determines the Presence of the Subcortical Maternal Complex in Pre-implantation Mouse Development.

B. Baibakov, L. Li, J. Dean; LCDB, NIDDK/NIH, Bethesda, MD

During oogenesis, at least four maternally-encoded proteins form a subcortical maternal complex (SCMC) that persists through pre-implantation development: FLOPED, MATER and TLE6 interact with each other while Filia binds independently to MATER. Genetic ablation of either Floped or Mater leads to disappearance of the complex from the subcortex and embryos derived from null females do not progress beyond 2-cells. After fertilization and the first cell division, the SCMC is normally excluded from regions of cell-cell contact and subsequently segregates to the 'outer' cells of the morulae. This exclusion, which is readily reversible upon disaggregation of blastomeres in the absence of calcium, results in the apparent absence of SCMC from the inner cell mass (epiblast) and its persistence in the trophectoderm of blastocysts. To determine if cell lineage or external location of a free cell surface dictates the presence of the SCMC, embryonic day 3.5 (E3.5) Tead4 null embryos were stained with antibodies to TLE6 and FLOPED. As reported (Development 134:3827, 2007), Tead4 null embryos do not form a trophectoderm, but remained as a clump of OCT4 positive cells, indicative of epiblast lineage. Immunolocalization of the SCMC with antibodies to TLE6 and FLOPED was restricted to 'outer' cells of E3.5 Tead4 null embryos. This indicates that topology rather than cell lineage determines the presence of the complex and that the SCMC is not sufficient to establish the trophectoderm.

2140/B608

Arginylation-Dependent Cell Migration Is Essential for Mouse Development.

S. Kurosaka[1], N. A. Leu[1], R. Bunte[2], S. Saha[1], C. Guo[3], W. He[4], A. Kashina[1]; [1]Animal Biology, University of Pennsylvania, Philadelphia, PA, [2]University Laboratory Animal Resources, University of Pennsylvania, Philadelphia, PA, [3]Janelia Farm, Ashburn, VA, [4]Gene Targeting and Transgenic Facility, University of Connecticut Health Center, Farmington, CT

Coordinated cell migration during development is crucial for morphogenesis and largely relies on cells of the neural crest lineage that migrate over long distances to give rise to organs and tissues throughout the body. Recent studies of protein arginylation implicated this poorly understood posttranslational modification in the functioning of cytoskeletal proteins, including actin, and in cell migration in culture. Knockout of arginyltransferase (Ate1) in mice leads to embryonic lethality and severe heart defects that are reminiscent of cell migration-dependent phenotypes seen in other mouse models. Ate1 knockout fibroblasts have abnormal cytoskeleton and lamella formation. Thus, arginylation is likely important to the general mechanisms of cell migration in culture and in embryogenesis. To test the hypothesis that arginylation regulates cell migration during morphogenesis, we produced Wnt1-Cre Ate1 conditional knockout mice (Wnt1-Ate1), with Ate1 deletion in neural crest cells driven by Wnt1 promoter. Wnt1-Ate1 mice die at birth and in the first 2-3 weeks after birth with severe breathing problems and growth and behavioral retardation, indicating the critical role of Ate1 in the functioning of the neural crest cells. Wnt1-Ate1 pups have prominent defects, including short palate and altered opening to the nasopharynx that likely contribute to the abnormal breathing and early death. Preliminary data suggest that other neural crest-dependent structures are affected, and that these effects are associated with the fastest migrating subpopulations of the neural crest cells. Analysis of cell movement in culture shows an overall delay in the migration of cells of the mesenchymal morphology that is likely regulated by intracellular mechanisms rather than extracellular signaling events. Taken together, our data suggest that arginylation plays a general role in the migration of the neural crest cells in development by regulating actin cytoskeleton and the molecular machinery that underlies cell migration through tissues and organs during morphogenesis.

2141/B609

Alteration of X-Linked Inhibitor of Apoptosis Expression Is Associated with Normal Placental Development.

S. Jeon[1], J. Jung[1], J. Lee[2], J. Park[3], G. Kim[1]; [1]Graduate School of Life Science and Biotechonology, Pochon CHA University, Seoul, South Korea, [2]Department of Molecular and Life Science, Pochon CHA University, Pochon, South Korea, [3]Department of Pharmacology, Seoul National University College of Medicine, Seoul, South Korea

Objective: The regulation of trophoblast apoptosis is essential for the normal placentation and increased apoptosis of placental trophoblast cause pregnancy pathologies such as intrauterine growth retardation (IUGR) and pre-eclampsia. The X-linked inhibitor of apoptosis (XIAP) is present in trophoblast during placental development; however, the accurate mechanism of XIAP related placenta development is still unknown. The aim of the study was to elucidate the function of XIAP on placenta including normal and pre-eclamptic placenta. Methods: A cross-sectional study was conducted to examine the expression pattern of XIAP in human placenta. Tissues were obtained from patients in the following groups: 1) term without labor (n=15); 2) term without labor pre-eclampsia (n=15); and 3) preterm without labor pre-eclampsia (n=11). The expression of XIAP was studied by quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) and Western blot. Non-parametric statistics were used for analysis. HTR-8 trophoblast cell line was exposed by 1% O2 hypoxic condition (24hrs and 48hrs.) and transfection of XIAP gene into HTR-8 cell line for 24 hrs and 48 hrs to examine whether they increased XIAP expression. RESULTS:1)Expression of XIAP is higher in peripheral region of normal placenta than pre-term and term pre-clamptic placenta ($P<0.05$). 2) The expression of XIAP in pre-term preeclampsia was increased than others.3) In vitro hypoxia induction in HTR-8 trophoblast cell line, the expression of XIAP was decreased and translocated from cytoplasm to nuclear.4) In hypoxic condition, the expression of XAIP was decreased followed by increased apoptosis-related genes were increased. CONCLUSION: Decreased XIAP cause apoptosis of trophoblast as well as alterative expression patterns and effects of XIAP is functionally involved in pre-eclampsia.

2142/B610

Serum Response Factor Is Required for Proper Morphogenesis of Mouse Cardiac Outflow Tract, Heart and Brain.

X. Hu, S. Zhang, R. Schwartz; IBT-Texas A&M University, Houston, TX

Serum Response Factor (SRF), a member of an ancient DNA binding family, is required for the transcriptional activation of various miRNAs and plays a particularly important role in smooth, skeletal and cardiac muscle development. Given the emerging roles of miRNAs in the developmental processes we hypothesized that the expression profile of cardiac neural crest-derived smooth muscle differs from either other types of smooth muscle, skeletal muscle or cardiac muscle. To test this hypothesis we inactivate SRF specifically in the region of Wnt1

expression by generating SRF Loxp/Loxp x Wnt1-Cre conditional knockout mice. We found that mutant embryos displayed a wide range of developmental abnormalities encompassing congenital heart diseases, cardiac outflow tract abnormalities, incomplete facial structure, neural tube disclosure and condensation. At E10.5 we show that 4th and 6th branchy arch arteries are hypoplastic and narrow while the 3rd is normal. At E11.5 we observed hemorrhage and edema in branchy arches, heart area and mid-hindbrain region in mutant embryos. At E12.5 we found developmental abnormalities of cardiac outflow tract, thinner and loose-connected heart walls with the attenuated level of smooth muscle a-actin(ACTA2). We profiled the expression of 385 miRNAs and validated the down expression of miR-181a, miR-181b and miR-720. Taken together, SRF is required for proper morphogenesis of mouse cardiac outflow tract, heart and brain.

2143/B611

Temporal Specific Deletion of Orc3 Gene Results in Ablation of Distinct Cell Types in the Mouse Cerebral Cortex.

Z. Huang[1], H. Kwon[1], H. Johng[1], K. Zang[2], L. F. Reichardt[2]; [1]Neurology & Anatomy, Univ. of Wisconsin Madison, Madison, WI, [2]Physiology, University of California San Francisco, San Francisco, CA

Orc3 is a core subunit of Origin Recognition Complex (ORC), a six-subunit protein complex conserved from yeast to human and essential for DNA replication and cell division in yeast and Drosophila. To study ORC function in mammals, we have generated a conditional knockout allele of the mouse orc3 gene. When orc3 was deleted from early developing cortex with an emx1-cre line, cortical development was severely affected, resulting in extensive loss of cortical and hippocampal structures at neonatal stage and reduced animal survival at postnatal to adult stage. Examination of embryonic brains revealed that cell proliferation in the cortical ventricular zone was severely reduced and radial glial progenitor cells severely depleted. These results indicate an essential role for orc3 in cell division in the developing mouse cortex. Interestingly, in the mutant embryonic cortex, a large number of early born neurons also underwent cell death, despite having apparently finished cell division. When orc3 was deleted at a slightly later stage with a nestin-cre line, reduced cell proliferation in the ventricular zone and diminished numbers of cortical radial glia were also observed, although the phenotypes were less severe compared with emx1-cre mutants and occurred at later stages. Most of the cortical and hippocampal structures were present at neonatal stage in nestin-cre mutants despite reduction of brain size. Surprisingly, severe hemorrhage was observed in the mutant neonatal cortex along dorsal midline, indicating a role for cells of the neural lineages in the development and/or maintenance of cortical blood vessels. Thus, our results indicate that ORC plays a conserved role in regulating cell division in mammals. The orc3 conditional allele also provides a useful tool for uncovering functions of different cell types during the development of mouse nervous and other systems.

2144/B612

Regulation of Focal Adhesion Complex Proteins in Developing Kidney.

H. Harn, C. Tsai, Y. Wang, M. Tang; Department of Physiology, National Cheng Kung University, Tainan, Taiwan

Substratum rigidity has been shown to downregulate the expression of focal adhesion complex proteins such as focal adhesion kinase (FAK), paxillin and talin. We found that FAK was initially degraded into 100 kDa, 90 kDa and 85 kDa N-terminal fragments from embryonic 19.5 day and subsequently the intact FAK was completely absent after birth in kidney. Other focal adhesion complex proteins such as paxillin, p130cas and cytosolic protein c-src were also downregulated during kidney development. To determine whether substratum rigidity affect expression of FAK, we employed primary culture of renal proximal tubular cells freshly isolated from 3-month-old rat kidney. Proximal tubular cells cultured on normal dish and collagen gel-coated dish, but not on collagen gel, exhibited focal adhesion complex proteins. The regulation of FAK was mediated by post-transcriptional mechanism as FAK mRNA level was not altered in developmental kidney or in primary proximal tubular cells cultured on different rigidity. Immunohistochemical study showed that FAK was present in interstitial cells, but absent in developing nephrons in embryonic kidney. After birth, FAK was completely absent in renal epithelium in cortex and expressed mostly in the nuclei of epithelial cells in inner medulla, indicating the role of 85 kDa species in nuclear translocation. We assessed kidney rigidity at different ages by dynamic mechanical analyzer (DMA) and atomic force microscopy. Our data showed that the rigidity of kidney was around 1 kPa 3 days after birth and increased gradually to 4 kPa within 2 months. However, the rigidity of embryonic and neonatal kidney was lower than the detection limit of DMA. Whether rigidity plays roles in the regulation of focal adhesion complex proteins in kidney remains to be investigated.

2145/B613

Increased Levels of O-GlcNAc Cause Disrupted Lens Fiber Cell Differentiation in Transgenic Mice.

K. Wang[1], S. Ho[2], W. Mao[3], P. Huang[4], F. Zhang[1], E. M. Schwiebert[2,5], J. E. Kudlow[1,2,5], A. J. Paterson[1,2,5]; [1]Medicine, Division of Endocrinology, Diabetes, and Metabolism, University of Alabama at Birmingham, Birmingham, AL, [2]Cell Biology, University of Alabama at Birmingham, Birmingham, AL, [3]Vision Sciences, University of Alabama at Birmingham, Birmingham, AL, [4]Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL, [5]Physiology and Biophysics, University of Alabama at Birmingham, Birmingham, AL

O-GlcNAcylation is an abundant and dynamic protein post-translational modification. For this modification, the monosaccharide N-acetylglucosamine (GlcNAc) is attached in a beta linkage to the side chain hydroxyl groups of specific serine or threonine residues on target proteins. Studies have shown that O-GlcNAc modification plays an essential role in regulating many important cellular processes such as; transcription, translation, signal transduction pathways, cell cycle control and cell division, and protein degradation. Thus it is reasonable to propose that O-GlcNAc is involved in the developmental process. The eye lens has been a popular system to study the fundamental process of development. This study has established a novel transgenic mouse model using the tetracycline inducible mouse system. The target transgene, GK-NCOAT, a dominant-negative form of NCOAT which suppresses removal of the O-GlcNAc residues from proteins, expresses specifically in lenses of gamma-F-crystallin-rtTA/TRE-GK-NCOAT bitransgenic mice after administration of doxycycline. We found that bi-transgenic lenses had higher O-GlcNAc modification levels. The lens fiber cell denucleation was inhibited and the lens epithelial cells formed multiple layers in bi-transgenic mice after administration of doxycycline. As we reported previously, O-GlcNAc is an endogenous inhibitor of the proteasome. We observed that the bi-transgenic lenses with administration of doxycycline had decreased proteasome activity.

Our study suggested that increased O-GlcNAc modification in the lens may inhibit lens fiber cell denucleation and that decreased proteasome activity might be a potential mechanism.

2146/B614

The Expression of CFTR in the Mouse Female Genital Tract during the Oestrus Cycle.

N. Mannowetz, G. Wennemuth; Saarland University, Inst. of Anatomy and Cell Biology, Homburg/Saar, Germany

Bicarbonate (HCO_3^-) is essential for enhanced sperm motility, capacitation and thus for egg fertilization. We have previously shown a resting flagellar beat frequency of 2-3 Hz in sperm bathed in buffer without HCO_3^-. Local perfusion of sperm with HCO_3^- (15 mM), leads to an accelerated beat frequency of 8-9 Hz. CFTR (cystic fibrosis transmembrane conductance regulator) is a chloride- and HCO_3^--conducting channel protein found - amongst other locations - in sperm and the female genital tract. Mutations of the CFTR gene lead to cystic fibrosis, an incurable worldwide disease strongly correlating with patients' infertility. Here we focus on the role of CFTR being essential for female fertility. Using qRT-PCR, we demonstrate by relative quantitation that CFTR transcripts are expressed differently in distinct segments of the female genital tract (vagina, uterus body, uterus horns, isthmus and ampulla). Additionally, we used pubescent (age 3 - 4 weeks) and superovulated mice to study whether CFTR transcript expression is regulated hormonally. The most striking effects occur in vagina and uterus body, with CFTR transcript expression values of 2.0 (se ±0.47) and 2.3 (se ±0.27) in superovulated mice. In pubescent mice, the expression pattern in these segments averages out at 0.8 (se ±0.23) and 1.0 (se ±0.14) respectively. In addition, we used an immunohistological approach to demonstrate the same expression variability of CFTR on protein level in pubescent and superovulated mice. We show that CFTR is localized in apical domains of the epithelium lining the female genital tract. Preliminary results demonstrate that the protein is expressed in both superovulated and pubescent mice. These findings illustrate that CFTR transcript expression is dependent on the hormone level during the oestrus peaking out at ovulation. We hypothesise that the epithelium of the female genital tract regulates HCO_3^- secretion during the oestrus to alter sperm motility.

2147/B615

Mouse Skeletal Muscle Differentiation Is Controlled in a Temperature-Dependent Manner.

A. Shima, R. Matsuda; Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Tokyo, Japan

Skeletal muscle differentiation is controlled by the myogenic regulatory factors (MRFs) MyoD, myf-5, myogenin and MRF4. To investigate the effect of low temperature on myogenesis, we cultured the mouse myoblast cell lines C2C12 and Sol8 at various temperatures. At 38°C, the myoblasts fused into multinucleated myotubes within 4 days after the induction of differentiation. Myotube formation was delayed but relatively normal at 35°C, whereas it was blocked at 30°C. The myoblasts cultured at 30°C expressed MyoD, which is required for myogenic determination, but not myogenin, which mediates terminal differentiation. Id3, which acts as a negative regulator of MRFs and decreases upon the initiation of differentiation, was continuously expressed at 30°C. The expression level of E2A, which acts as a positive regulator of MRFs, exhibited no difference at both temperatures. We also found that the expressions of the muscle-specific microRNAs miR-1, miR-133a, miR-181a and miR-206 were not upregulated at 30°C. These low temperature-induced inhibitions of myogenic differentiation were canceled in the presence of growth factor X and L-ascorbic acid phosphate (stabilized vitamin C). Myoblasts cultured in the medium containing growth factor X and L-ascorbic acid phosphate at 30°C expressed myogenin and then fused into multinucleated myotubes expressing sarcomeric myosin heavy chain. In addition, we investigated the expressions of MyoD and myogenin during mouse satellite cell activation in single fiber culture as an in vivo model and found that the expression of myogenin, but not of MyoD, was inhibited at 30°C. These results suggest that the mouse skeletal muscle formation can be regulated by temperature, and that the physiological body temperature plays an important role in the myogenesis of homothermal animals.

2148/B616

Effect of Mechanical Stretching on Expressions of Muscle Specific Transcription factors in Proliferated Myoblasts.

S. Abe, O. Iwanuma, Y. Ide; Anatomy, Tokyo Dental College, Chiba, Japan

Recent studies showed that application of mechanical stretching to myocyte induced cell proliferation and had an influence on the muscle structural protein myosin heavy chains. Based on these reports, it has now been clear that mechanical stress on myocyte promotes not only cell proliferation but also changes the characteristics of the cells. However, how these alterations happen and which molecule's expression influences these changes remains largely unknown. Although roles of muscle-specific molecules have been known during activation, proliferation and differentiation of myoblast precursor cells, i.e. satellite cells, there has been no report regarding with their expression level for the cells, which were exposed to mechanical stretching. Thus in the current research, we examined expression of four important members of MRFs in the myoblasts both at mRNA and protein levels, which were subjected to mechanical stretching in in vitro condition. Our results showed that MyoD expression existed both in the stretch and in the control group at all time periods of the mechanical stimulus. Myf-5 expressed only at early stage of the stretch group. Although mRNA and protein expressions of myogenin and MRF4 were detected both in the stretch and in the control group at 12 hours after the stretching, their expressions were only shown in the stretch group at 24 hours after the mechanical stimulus. However, at 36 and 48 hours, none of the MRFs examined except MyoD appeared in both groups. Our results suggest that the MRFs are up regulated upon mechanical stimulus and each member plays a different major role for either proliferation or differentiation of the myoblasts.

2149/B617

A Novel Role of the Mad Family Member Mxd3 in Proliferation of Cerebellar Granule Neuron Precursors.

J. Yun, J. Rust, T. Ishimaru, E. Diaz; Pharmacology, UC Davis, Davis, CA

Granule neurons are the major cell type of the cerebellum; thus, granule neuron development is important for the function of the cerebellum in motor coordination. Through an expression profiling approach, MAX dimerization protein3 (Mxd3) was identified as a candidate gene implicated in granule neuron progenitor (GNP) proliferation. Mxd3 is a member of the Mad family of basic helix-loop-helix/leucine zipper (bHLHZ) transcription factors which are generally thought to function as an antagonist of Myc. We present evidence supporting a novel role

of Mxd3 in sonic hedgehog (Shh)-mediated proliferation of GNPs. Mxd3 mRNA and protein are expressed in the outer EGL in which proliferating GNPs reside during early development, with an expression pattern similar to that of N-myc, a key factor in the Shh signaling pathway. Moreover, Mxd3 is expressed mainly during the S-phase of the cell cycle in GNPs. Medulloblastomas derived from patched heterozygous mice show strong expression of Mxd3. Interestingly, treatment of purified GNPs with Shh induces expression of Mxd3 via the Smoothened receptor. Overexpression studies in cultured GNPs provide evidence that Mxd3 is sufficient to induce proliferation of GNPs in the absence of Shh. Furthermore, knockdown experiments showed that Mxd3 is required for Shh-mediated proliferation of GNPs. Surprisingly, basic-domain-dependent DNA binding of Mxd3 is not required for its proliferative effect, suggesting that Mxd3 interacts with other DNA binding proteins to repress transcription. Taken together, these results support a novel role for Mxd3 in cerebellar GNP proliferation and possibly tumorigenesis, and they challenge the current paradigm that Mxd3 should antagonize N-myc by competition for direct DNA binding via Max dimerization.

2150/B618

A Neural Crest Screen Identifies an Amino Acid Essential for Erbb3 Function.

K. Buac[1,4], D. Watkins-Chow[1], E. Escobar[2], M. Jones[3], W. Pavan[1]; [1]Genetic Disease Research Branch, National Human Genome Institute, Bethesda, MD, [2]Transgenic Mouse Core, NHGRI, Bethesda, MD, [3]Genomics Core, NHGRI, Bethesda, MD, [4]The George Washington University, Washington, DC

The neural crest (NC) is a population of embryonic stem cells that gives rise to numerous cell types, including the glia and neurons of the peripheral and enteric nervous systems, and melanocytes of the skin and hair. Mutations in genes and genetic pathways regulating NC development lead to a wide spectrum of human developmental disorders, collectively called neurocristopathies. To identify molecular pathways regulating NC development and understand how alterations in these processes lead to disease, we established an N-ethyl-N-nitrosourea (ENU) mutagenesis screen utilizing a mouse model sensitized for NC defects, Sox10LacZ/+. One of the lines identified, called modifier of Sox10 expression pattern 1 (msp1), shows reduced Sox10LacZ expression in cranial ganglia, cranial nerves and the sympathetic chain; however, the development of other Sox10-expressing cells appears unaffected by the mutation. Linkage analysis, sequencing, and complementation testing confirmed that msp1 is a new allele of the receptor tyrosine kinase Erbb3, Erbb3msp1, that carries a single amino acid substitution in the extracellular region of the protein. The ENU-induced mutation does not alter protein expression, however, is sufficient to impair ERBB3 signaling, such that the embryonic defects observed in msp1 resemble those of Erbb3 null alleles. Biochemical analysis of the mutant protein showed that ERBB3 is expressed on the cell surface, but its ligand-induced phosphorylation is dramatically reduced by the msp1 mutation. These findings highlight the importance of the mutated residue for ERBB3 receptor function and activation and underscore the utility of using an ENU mutagenesis to dissect the roles of discrete protein domains.

2151/B619

Detection of Estrogen Mimics in Bamboo: Indirect Evidence of Phytosterol Presence As Determined By Radioimmunoassay and Competitive Estrogen Receptor Binding Assay Procedures.

E. E. Trueblood[1], B. Baldwin[4], A. Kouba[3], G. Thibaudeau[1], S. Willard[2]; [1]Biological Sciences, Mississippi State University, Mississippi State, MS, [2]Biochemistry, Mississippi State University, Mississippi State, MS, [3]Memphis Zoo, Memphis, TN, [4]Plant and Soil Sciences, Mississippi State University, Mississippi State, MS

Phytoestrogens, naturally occurring plant compounds that possess estrogenic activity, have been linked to decreased fertility in animals through the disruption of the development, differentiation, and/or subsequent function of the reproductive system. It has been hypothesized that some species of bamboo contain hormone mimics that could negatively impact giant panda (Ailuropoda melanoleuca) reproduction, yet little information is available regarding the presence or physiological relevance of phytosterols in bamboo species. In the current investigation, four species of bamboo, including Phyllostachys aurea and P. aureosulcata which are both major components of captive giant pandas diets in the U.S., were examined for the presence of phytoestrogenic activity using two methods: an estrogen antibody-dependent immunoreactive cross-reactivity assay and an estrogen receptor binding assay. The samples were collected from growth plots and processed (dried, ground and extracted in methanol) for detection via an estradiol radioimmunoassay (RIA) and recombinant estrogen receptor alpha and beta competitive binding assay (ERBA). The RIA data (of concentrated extracts) revealed significant differences ($P < 0.05$) in immunoreactive estrogenic activity between the leaf (20.3 ± 1.9 pg/ml), branch (15.9 ± 0.9 pg/ml) and culm (11.4 ± 0.7 pg/ml), with the leaf extracts exhibiting the greatest concentrations and P. glauca having the greatest estrogenic leaf content (22.5 ± 5.1 ng/ml) compared to the other bamboo species. Detection of binding ability to the mammalian estrogen receptor via ERBA revealed that although all bamboo parts bound both the alpha and beta estrogen receptors at various concentrations, the leaf tissue had the greatest ($P < 0.05$) binding affinity as compared to the branch and culm at similar dilutions of concentrated extracts. These results present indirect evidence of the presence of phytoestrogenic mimics in bamboo and require further study to substantiate. Whether phytosterols in bamboo have physiological relevance to giant panda physiology also remains to be determined.

2152/B620

"Human Cardiomyocyte Stem Cells as an Alternative to Cardiac Tissue Transplant".

J. Sharma, M. Majdoub, N. Liles, N. Taw, G. Velazquez, C. Sharma, M. Sharma, C. DiMaggio; Celprogen, San Pedro, CA

In the past few years it has been established that the heart contains a reservoir of stem and progenitor cells. These cells are positive for various stem/progenitor cell markers (c-Kit, Sca-1, and P-glycoprotein) properties). The adult human heart contains small populations of indigenous committed cardiac stem cells (CSC) or multipotent cardiac progenitor cells, identified by their cell-surface expression of c-kit (the receptor for stem cell factor), P-glycoprotein (a member of the multidrug resistance protein family), and Sca-1 (stem cell antigen 1, a mouse hematopoietic stem cell marker) or a Sca-1-like protein. Cardiac stem cells represent a logical source to exploit in cardiac regeneration therapy because, unlike other adult stem cells, they are likely to be intrinsically programmed to generate cardiac tissue in vitro and to increase cardiac tissue viability in vitro. Cardiac stem cell therapy could, therefore, change the fundamental approach to the treatment of heart disease. Cellular cardiomyoplasty may indeed be a novel method to regenerate damaged myocardium. Recently, we have successfully isolated CSCs

from small biopsies of human myocardium and expanded them ex vivo by many folds without losing differentiation potential into cardiomyocytes and vascular cells, bringing autologous transplantation of CSCs closer to clinical evaluation. Our method for ex vivo expansion of resident CSCs for subsequent autologous transplantation back into the heart, may give these cell populations, the resident and the transplanted one, the combined ability to mediate myocardial regeneration to an appreciable degree, and may change the way in which cardiovascular disease will be approached in the future.

2153/B621

A Mechanism Linking Lipid Dynamics and Longevity.

A. Goldberg, C. Gregg, T. Boukh-Viner, P. Kyryakov, S. Bourque, G. Machkalyan, H. Mashhedi, S. Milijevic, A. Hossain, S. Lo, M. A. London, J. M. Lee, V. Richard, V. Titorenko; Biology Department, Concordia University, Montreal, QC, Canada

The fundamental mechanisms of aging are conserved across phyla. We use the yeast Saccharomyces cerevisiae as a model to study the mechanisms of cellular aging in multicellular eukaryotes. Our objective is to establish the mechanisms underlying the essential role of lipid dynamics in regulating longevity. Yeast aging can be slowed down by calorie restriction, a low-calorie dietary regimen that extends life span and delays age-related disorders in a wide spectrum of organisms. We assessed the effect of a calorie restriction diet and numerous mutations extending yeast life span on the spatiotemporal dynamics of the proteomes and lipidomes of organelles involved in lipid metabolism. These organelles include the endoplasmic reticulum (ER), peroxisomes and lipid bodies. We also examined the spatiotemporal dynamics of organellar proteomes and lipidomes in long-lived yeast mutants impaired in various pathways of lipid metabolism and transport. Our findings revealed a mechanism linking longevity and lipid dynamics. In this mechanism, a calorie-rich diet suppresses peroxisomal oxidation of free fatty acids (FFA) that originate from neutral lipids synthesized in the ER and deposited within lipid bodies. The resulting accumulation of arrays of FFA within lipid bodies initiates several negative feedback loops regulating the metabolism of neutral lipids. Due to the action of these negative feedback loops, aging yeast accumulate diacylglycerol (DAG). The buildup of monounsaturated FFA promotes lipoapoptosis (a caspase- and mitochondria-independent form of programmed cell death), whereas the accumulation of DAG triggers a protein kinase C-dependent signal transduction network affecting multiple stress response-related processes. Implementing our understanding of the mechanism linking longevity and lipid dynamics, we developed a life-span assay that was used for a high-throughput screening of extensive compound libraries. We identified five groups of novel anti-aging small molecules that significantly delay yeast aging by remodelling lipid dynamics in the ER, peroxisomes and lipid bodies and by promoting “mitohormesis” through the activation of a distinct set of stress response-related processes in mitochondria.

2154/B622

Induction of the Osmotic Stress Response Extends Life Span in Caenorhabditis Elegans.

E. Smith[1,2], A. T. Le[1], M. Kaeberlein[1]; [1]Pathology, University of Washington, Seattle, WA, [2]Cellular and Molecular Biology, Northwestern University, Chicago, IL

Survival in the natural environment is limited by many factors including environmental stressors such as thermal stress, oxidative stress, UV stress, and osmotic stress. Interventions that increase life span in a laboratory setting tend to increase stress resistance as well. The converse can also be true. For example, induction of the heat shock response in C. elegans extends life span (Rea et al, 2005). In yeast, exposure to osmotic stress increases life span (Kaeberlein et al, 2002). In this study, we find that induction of the osmotic stress response, either environmentally or genetically, increases longevity in C. elegans. The median life span of worms maintained on plates containing high peptone or sorbitol is increased by 25% or 38%, respectively. This life span extension does not require the FOXO transcription factor, Daf-16. We find that both peptone and sorbitol induce the osmotic stress response using a gpdh-1::GFP reporter, indicating upregulation of glycerol biosynthesis. In animals that have a constitutively active osmotic stress response, osr-1, life span is extended by 15% compared to wild-type animals on normal media. Combined, these results indicate that induction of the osmotic stress response is sufficient to extend life span in C. elegans. Future studies will be aimed at trying to understand the mechanism by which induction of the osmotic stress response increases longevity.

2155/B623

Inhibition of eIF2Bδ/F11A3.2 during Adulthood Extends Lifespan in Caenorhabditis Elegans.

D. Tohyama[1], A. Yamaguchi[2], T. Yamashita[3,4]; [1]Department of Neurobiology, Graduate School of Medicine, University of Chiba, Chiba, Japan, [2]Department of Neurobiology, Graduate School of Medicine, University of Chiba, Chiba, Japan, [3]Department of Molecular Neurosciece, Graduate School of Medicine, Osaka University, Osaka, Japan, [4]Department of Neurobiology, Graduate School of Medicine, University of Chiba, Chiba, Japan

The critical role of protein synthesis in regulating lifespan has been evidenced. This study shows that adult-onset RNAi inactivation of eukaryotic initiation factor 2Bδ (eIF2Bδ/F11A3.2), a subunit of eIF2B, extends the mean lifespan of Caenorhabditis elegans. eIF2B is a GDP-GTP exchange factor for eIF2—a rate-limiting factor for protein synthesis initiation. 35S-Methionine incorporation assay showed that global protein synthesis is reduced by eIF2Bδ/F11A3.2 RNAi. Inhibition of eIF2Bδ/F11A3.2 during adulthood conferred thermal and oxidative stress resistance, and reduced the fecundity and fat storage, suggesting the possible trade-offs of resources between reproduction and somatic maintenance. Lifespan extension by adult-onset eIF2Bδ/F11A3.2 RNAi is suppressed in FOXO transcription factor daf-16 deletion mutants. Adult-onset eIF2Bδ/F11A3.2 RNAi increases the expression of stress-resistant genes including hsp-16.2, hsp-70, hsp90, and sod-3, some of which are reported to be targets of DAF-16. Adult-onset eIF2Bδ/F11A3.2 RNAi in daf-16 mutants reduced fecundity, but did not extend lifespan. Furthermore, adult-onset eIF2Bδ/F11A3.2 RNAi did not extend the lifespan of germline-defective glp-4 organisms. Thus, it is possible that eIF2Bδ/F11A3.2 RNAi during adulthood prolongs lifespan via daf-16, which induces stress resistance in organisms. This might be the mechanism, at least in part, for trade-offs of resources between reproduction and somatic maintenance.

2156/B624

Expression, Subcellular Localization, and Antioxidant Role of Mammalian Methionine Sulfoxide Reductases in Saccharomyces

Cerevisiae.

G. Kwak, J. Kim, H. Kim; Biochemistry and Molecular Biology, Aging-associated Vascular Disease Research Center, Yeungnam University College of Medicine, Daegu, South Korea

Methionine sulfoxide reductases, MsrA and MsrB, are key enzymes that repair oxidatively damaged proteins by reducing oxidized methionines back to methionines in a stereospecific manner. In contrast to the growing evidence of a role for MsrA in antioxidant defense, little is known about the role of MsrB in cellular protection against oxidative stress. In this study, we overexpressed mammalian MsrB and MsrA genes in Saccharomyces cerevisiae and investigated their subcellular localization and antioxidant roles. We report the first evidence that overexpression of MsrB3 in yeast cells protected them against H2O2-mediated cell death. In addition, MsrB2 overexpression also provided yeast cells with resistance to oxidative stress, as did the overexpression of MsrA. We found that the mitochondrial MsrB3 protein (MsrB3B) was localized to the cytosol but not to the mitochondria of the yeast cells. The mitochondrial MsrB2 protein was found to be present in mitochondria and to a lesser extent the cytosol of the yeast cells. Our results emphasize that mammalian MsrB and MsrA proteins play important roles in the protection against oxidative stress and demonstrate a broad substrate specificity, functioning in both mammals and lower eukaryotic yeast cells.

2157/B625

Cell Cycle-Regulated Localization of a Class of Transmembrane Proteins in Saccharomyces Cerevisiae.

A. Eldakak, R. Li; Stowers Institute for Medical Research, Kansas City, MO

The budding yeast Saccharomyces cerevisiae reproduces by asymmetric cell division to give rise of a young daughter cell by an aged mother cell. Polarization orchestrates this asymmetric division so the new born bud will inherit all the vital requirements for its own growth. Despite mother's age, the newly produced buds have full capacity of life span indicating that their ages are reset through unknown mechanism. It's known that age asymmetry depends on retaining damaged protein and ERC (ExtraChromosomal Circles) in the mother cell which is important for the fitness of the newly born cells. Surprisingly, we found a group of transmembrane proteins, mostly involved in polyamines and amino acids transport, to be asymmetrically localized between the mother and the bud in a cell-cycle dependent manner. Our studies on Tpo1p, a transporter involved in excretion of polyamines, revealed that this asymmetric localization is independent of the septin barrier but requires the actomyosin-based transport system. To search for genes required for this asymmetric inheritance, a screen was conducted using the yeast knockout library looking for mutations that will allow the cells to grow on a lethal dose of the polyamine analogue, MGBG (methylglyoxal bisguanylhydrazone). Those mutations that confer higher resistance to MGBG were screened for a disruption in the asymmetric localization of Tpo1p. Among those identified in this screen are genes involved in ubiquitination, gene transcription, phosphorylation, mitochondria, protein trafficking and cytoskeleton, which reflects the complexity that governs this asymmetric inheritance. Our work hypothesize that this group of transporters could work as aging determinants that, through a novel retention mechanism, are sequestered in the mother cell while a new wave of expression allows their targeting only to the newly born cell.

2158/B626

The Forkhead Transcription Factor FOXJ1 Is Required for Specification and Establishment of the Adult Neural Stem Cell Niche.

B. Jacquet, T. Ghashghaei; North Carolina State University, Raleigh, NC

In the developing central nervous system, the periventricular region acts as an important site of neuronal proliferation and specification. In adults, however, only the dentate gyrus of the hippocampus and the anterior subventricular zone (aSVZ) remain neurogenic. Transcription factors are key regulators of patterning and tissue specification. Transcription factors that specify the adult neural stem cell niche have remained largely unknown. Here, we report that the forkhead transcription factor FoxJ1 is required for establishment and maintenance of the adult neural stem cell niche cell autonomously, and regulation of progenitor activity non-autonomously. The complete absence of FOXJ1 activity in the CNS results in a disrupted neurogenic niche, with a pronounced layer-specific dysgenesis in the olfactory bulbs. Intriguingly, nearly all the phenotypes associated with deletion of FOXJ1 emerge perinatally, which signifies the role of FOXJ1 as an important temporal regulator of brain development. Our results, for the first time, identify a transcription factor with a temporally restricted role in specification and establishment of the adult neural stem cell niche, as well as in maintenance of postnatal and adult neurogenesis in the olfactory bulbs.

Bacterial Development Programs: Quorum Sensing, Sporulation, and Beyond (2159 – 2162)

2159/B627

Functional and Mechanistic Characterization of Toxin Antitoxin Module Implicated in Programmed Cell Death of Bacillus Anthracis.

S. Agarwal, R. Bhatnagar; School of Biotechnology, Jawaharlal Nehru University, Delhi, India

Bacterial system has an astounding capacity to develop resistance determinants in a short span of time leading to multidrug resistance. Therefore, it is of paramount importance to develop newer antibiotics with greater potential and fewer side effects against bacterial aggression. Comparative genome analysis of *Bacillus anthracis* revealed a pair of linked genes encoding *pemK* (K, killer protein) and *pemI* (I, inhibitory protein) homologous to pem loci of other organisms. Expression of PemK in *Escherichia coli* and *Bacillus anthracis* was bacteriostatic whereas the concomitant expression of PemI reversed the growth arrest. PemK expression effectively inhibited protein synthesis with no effect on DNA replication. Ribosome binding potential of PemK further confirmed its role in translation attenuation. Chimeric oligonucleotides with a specific ribonucleotide dually labeled with 6-FAM on 5-end and BHQ1 at 3-end established the specificity of PemK as a sequence specific endoribonuclease. Site directed mutagenesis demonstrated the role of His59, Glu78, Gln21 and Glu79 in catalysis. Co-expression and interaction of these proteins confirmed it to be a prototypical Type II addiction module. Their *in vitro* interaction was determined employing fluorescence polarization, ANS binding, circular dichroism, native PAG, far western blotting and acrylamide

quenching. The stoichiometery of interaction was determined as 1:1. Thermal and chemical denaturation analysis reflected poor conformational stability of PemI as compared to PemK. Circular dichroism analysis indicated that PemI contains twice the amount of β-sheets as PemK. Transcriptional fusions and gel retardation assays demonstrated that PemI binds to its upstream DNA sequence. The toxin-antitoxin complex retained the activity to bind to the promoter/regulatory region upstream of the operon and demonstrated cooperativity in autoregulation. The role of these genes in bacterial stress physiology was also elucidated. This study reports the first evidence of an active chromosome encoded Toxin-Antitoxin locus in *Bacillus anthracis*.

2160/B628

Caulobacter Requires a Dedicated Mechanism to Initiate Chromosome Segregation.

E. Toro[1], S. Hong[1,2], H. H. McAdams[1], L. Shapiro[1]; [1]Developmental biology, Stanford University, Redwood city, CA, [2]Physics, Stanford University, Stanford, CA

The Caulobacter chromosome is highly organized within the cell. The distance of any given DNA locus with respect to the pole is reproducible from one cell to another, and during segregation loci are rapidly and sequentially separated, suggesting that a dedicated mechanism contributes to this process. Recent theoretical and experimental work, however, has called into question the assumption that a dedicated machine is required for bacterial chromosome segregation. We use chromosomal inversions, targeted mutagenesis, and in vivo time-lapse imaging to show that the ancestral parS sequence serves as the site of force exertion for the initiation of segregation in Caulobacter, and that ParA activity is necessary for this movement. When the parS sequence is moved farther from the origin, the cell waits for parS to be replicated before segregation is initiated. Also, in the absence of parS-directed movement, segregation of newly replicated loci does not begin. These results show that Caulobacter requires a dedicated mechanism to initiate chromosome segregation, but do not preclude a role for forces exerted by DNA/RNA polymerases, entropic exclusion, or condensing compaction during bulk DNA movement. Finally, we demonstrate that the parS sequence is specifically targeted to the cell pole while the origin of replication is not, and provide evidence suggesting that the Caulobacter chromosome is "clocked" with respect to the parS centromere. The Caulobacter parABS system and its associated proteins are thus emerging as the functional equivalent of a eukaryotic kinetochore, as they initiate segregation, orient and anchor the chromosome, and signal the onset of segregation so that cytokinesis may begin.

2161/B629

Environmental Sensing by Escherichia Coli.

H. Tuson, D. Weibel; Biochemistry, University of Wisconsin - Madison, Madison, WI

Cellular mechanisms of surface sensing and differentiation are important in a variety of bacterial processes ranging from plant pathogenesis to oral plaque formation to catheter-associated infections. These mechanisms are also fundamental to swarming, in which bacterial cells on surfaces elongate into multinucleate filaments and over-express flagella. Populations of swarming cells display coordinated growth and motility that may be important in colonizing new niches. Previous work by McCarter et al. has demonstrated that the viscosity of the fluid in direct contact with cells of *Vibrio parahaemolyticus* can trigger the differentiation of cells into the swarming phenotype. This remarkable process requires the polar flagellum, which relays information on the extracellular environment to processes inside of the cell. In this work we focus on dissecting the sensor and stimuli for differentiation in swarming strains of *Escherichia coli* using materials science to control the cellular microenvironment—that is, the chemical and physical region that is sensed by a cell, and is typically defined by molecular contact, mass transport, and diffusion. In contrast to *V. parahaemolyticus*, the viscosity of the fluid surrounding cells of *E. coli* has no appreciable affect on their differentiation into swarmer cells. Furthermore, aflagellate cells of swarming strains of *E. coli* retain their ability to differentiate on agar surfaces, indicating that *E. coli* uses a different external cue and sensory mechanism for triggering differentiation. To study differentiation more systematically, we synthesized a series of polyacrylamide hydrogels that have tunable chemical and physical properties, and studied differentiation on these materials. In this poster we describe the relationship between the chemistry and properties of hydrogels that stimulates cellular differentiation—and, in some cases, hyperdifferentation—and suggest a mechanism for surface sensing in swarming strains of *E. coli* that is supported by observations of the physiologic response of strains with key mutations. This approach provides a testing ground for understanding how bacterial cells sense their environment and how perturbations reprogram physiology and behavior.

2162/B630

Redox-Active Antibiotics Control Gene Expression and Community Behavior in Divergent Bacteria.

T. K. Teal[4], A. Price-Whelan[4], D. K. Newman[1,2,3], L. Dietrich[1]; [1]Department of Biology, M.I.T., Cambridge, MA, [2]Department of Earth and Planetary Science, M.I.T., Cambridge, MA, [3]Howard Hughes Medical Institute, M.I.T., Cambridge, MA, [4]Division of Biology, California Institute of Technology, Pasadena, CA

A variety of bacteria are known to release colorful, redox-active pigments. Generally these have been regarded as antibiotics that serve the sole purpose of inhibiting competing organisms. Using RNA arrays we found that one such compound, the endogenous antibiotic pyocyanin from *Pseudomonas aeruginosa*, acts as a signaling molecule that activates the transcription factor SoxR. In *Escherichia coli*, SoxR regulates the superoxide stress response. Surprisingly, bioinformatic analysis coupled with gene expression studies in *P. aeruginosa* and *Streptomyces coelicolor* revealed that the majority of SoxR regulons in bacteria lack the genes required for stress responses despite the fact that many of these organisms still produce redox-active small molecules. This finding indicated that redox-active pigments play a role independent of oxidative stress. Using *P. aeruginosa* and *S. coelicolor* mutants that are defective in biosynthesis of these pigments, we found that they have profound effects on the structural organization of colony biofilms: While wild-type cells form smooth colonies when spotted onto agar plates, the absence of pigments causes the cells to aggregate into visually striking, wrinkled morphologies. Our results show that redox-active pigments are not merely virulence factors or antibiotics, but also play important, conserved roles in the developmental process of bacterial communities.

Development and Carcinogenesis (2163 – 2167)

2163/B631

Asymmetric Localization of the Protein Kinase aPKCζ Regulates Neural Progenitor Adhesion and Proliferation.

S. Ghosh[1,5], T. Marquardt[2], J. P. Thaler[3], S. Andrews[4], K. Kislin[5], E. Rushing[6], K. Aldape[7], W. McDonough[5], M. Berens[5], S. L. Pfaff[4], T. Hunter[4]; [1]Basic Medical Sciences, University of Arizona College of Medicine-Phoenix, Phoenix, AZ, [2]European Neurosciences Institute, Gottingen, Germany, [3]University of Washington, Seattle, WA, [4]Salk Institute for Biological Studies, La Jolla, CA, [5]Translational Genomics Research Institute, Phoenix, AZ, [6]Armed Forces Institute of Pathology, Washington D.C., DC, [7]University of Texas M.D. Anderson Cancer Center, Washington D.C.

Neural stem cells (NSC) give rise to all the cell types of the central nervous system (CNS). In the neurogenic phase of CNS development, the proliferating progenitors are found medially within the neuroepithelium. The adherens junctions on the apical membrane of these cells allow for cell-cell adhesion and medial stratification. In contrast, differentiating neuronal precursors delaminate and migrate laterally, establishing the laminar layers. Apical adherens junctions also establish the apical-basal polarity in neural progenitors, which in turn is postulated to lead to asymmetric inheritance of cell fate determinants during neurogenic divisions. The signaling pathways and cellular mechanisms that regulate the assembly and asymmetric localization of adherens junctions in neural progenitors remain elusive. Here we show that atypical protein kinase C ζ/λ (aPKCζ/λ) localizes at the apical membrane of proliferating neural stem cells in developing chick neural tube. Disrupting the endogenous localization of this kinase results in loss of apical cell adhesion junctions, and the increased proliferation and dispersion of neural progenitors into the differentiated cell layers. Thus, the precise subcellular compartmentalization of aPKCζ/λ within neural progenitors provides an instructive signal for the apical assembly of adherens junctions. Apical aPKCζ coordinates neural stem cell proliferation and the overall stratification of cell types within the neural tube. In light of this fundamental role of this kinase, can abnormal aPKCζ/λ function be associated with tumors of the CNS? The intrinsic proliferative potential of stem cells, if not tightly regulated may predispose them to transform into cancer stem cells. Indeed, recent studies demonstrate that gliomas contain a subpopulation of cancer cells with stem cell characteristics and that these may arise from NSC. We have demonstrated that aPKCζ/λ expression is significantly enhanced in tumor-cells in clinical Glioblastoma multiforme (GBM) and the knockdown of aPKCζ/λ in a GBM-derived cell line decreases cell migration. Our studies suggest that aPKCζ/λ, a protein kinase and thus a highly attractive target for rational drug design, may be a novel therapeutic target in GBM.

2165/B633

A Chemical Genetic Screen of the Wnt Pathway Identifies a Potent and Specific Inhibitor.

C. Thorne[1], E. Tahinci[1], K. Meyers[1], J. Schneider[1], B. LaFleur[2], D. Miller[1], E. Lee[1]; [1]Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN, [2]Section of Biostatistics, University of Utah, Salt Lake City, UT

Wnt/β-catenin signaling plays a prominent role in metazoan development and adult stem cell maintenance. Inappropriate activation of the canonical Wnt pathway is a critical event in the genesis of common human cancers and birth defects. The core cytoplasmic event is the regulation of the multicomponent β-catenin destruction complex, consisting of glycogen synthase kinase 3 (GSK3), adenomatous polyposis coli (APC), and the scaffold protein axin. In the absence of a Wnt ligand, this complex is active and stimulates the phosphorylation of β-catenin, leading to its degradation. Upon receptor activation, the complex is inactivated resulting in stabilization of β-catenin and, ultimately, degradation of axin. Consequently, β-catenin translocates to the nucleus and activates a TCF/Lef-dependent transcriptional program. We performed a forward chemical genetic screen that recapitulates activation of the canonical Wnt pathway by the Wnt coreceptor LRP6 using Xenopus egg extracts. Addition of β-catenin-firefly luciferase and axin-Renilla luciferase fusion proteins to LRP6-activated egg extracts allowed us to screen for regulators of β-catenin and axin turnover. In this study we characterize the most potent hit from the screen, VU-WS30, as a specific Wnt/β-catenin pathway inhibitor in vitro and in vivo. In cultured cells, VU-WS30 blocks accumulation of β-catenin and promotes the stabilization of axin, resulting in inhibition of Wnt/β-catenin-mediated gene transcription with an EC50 of 10 nM. VU-WS30 has activity in vertebrate and invertebrate model organisms, indicating that its target is conserved. Significantly, we find that VU-WS30 inhibits Tcf/Lef transcription in the context of mutations of APC and β-catenin commonly found in hereditary and sporadic cases of colorectal cancer. We predict that VU-WS30 will be a valuable pharmacological agent to study Wnt signaling in animal development, and that it represents a potential lead compound for the treatment of Wnt-driven cancers.

2166/B634

Subtelomeric DNA Methylation and Telomere Length in Human Hepatocarcinogensis.

T. Um[1, 2, 3], B. Oh[4], Y. Park[1, 2, 3]; [1]Department of pathology, Yonsei University College of Medicine, Seoul, South Korea, [2]Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, South Korea, [3]Innovation Cluster for Bio-Fusion Industry based on Nano Technology (ICBIN) and Center for Chronic Metabolic Disease, Yonsei University College of Medicine, Seoul, South Korea, [4]Cancer Metastasis Research Center, Yonsei University College of Medicine, Seoul, South Korea

Subtelomeric chromatin modifications are known to be important regulators for telomere length. We examined subtelomeric DNA methylation status in 32 pairs of hepatocellular carcinomas (HCCs) and adjacent non-HCCs, and subsequently analyzed their relation to telomere length. Subtelomeric DNA methylation was examined at chromosome 7q (104 kb), 8q (27 kb), 17q (4 kb), 18p (2 kb), 21q (31 kb) and XpYp (40 kb), using methylation-specific PCR (distance to telomere indicated in parenthesis). Telomere length of tissue samples was determined by Southern blotting. Methylation pattern was various at region to region; chromosome XpYp revealed high incidence of full-methylation in both HCCs and non-HCCs whereas un-methylation was dominant at chromosomes 8q and 21q. Thirty HCCs (30/32) showed different methylation pattern at least one site compared to adjacent non-HCCs. Hyper- and hypo-methylation in HCCs compared to adjacent non-HCCs was found in the incidence of 42 and 21 out of 192, respectively, and hypermethylation in HCCs was more frequent events than hypomethylation in those (p=0.008). HCCs with long telomeres (n=15, longer than mean telomere length of HCC, 8.17 kb) showed higher rates of methylation pattern changes compared to those with short telomeres (n=17) (p=0.028), and a similar pattern of result was found in HCCs with lengthen telomeres, which had longer telomeres than adjacent non-HCCs, although it was statistically insignificant. In summary,

subtelomeric chromatin structure varies at region to region. Methylation status is frequently altered during hepatocarcinogenesis, indicating that dynamic regulation of subtelomeric chromatin structure exists in hepatocarcinogensis. Furthermore, changes in subtelomeric chromatin structure are suggested to be influential on regulation of telomere length in hepatocarcinogenesis.

2167/B635

Identification of Critical Cancer-Related Genes Based upon Epigenetic Alterations Patterns of DNA Methylation during Hepatitis B Virus Related Human Multistep Hepatocarcinogenesis.

H. Kang[1], T. Um[1], B. Oh[2], Y. Park[1]; [1]Department of Pathology, Center for Chronic Metabolic Disease, Institute of Gastroenterology, Brain Korea 21 Project for Medical Science, Yonsei University College of Medicine, Seoul, South Korea, [2]Department of Pathology, Cancer Metastasis Research Center, Yonsei University College of Medicine, Seoul, South Korea

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and hepatitis B virus (HBV) is one of main causes. Epigenetic silence of tumor suppressor genes has been reported to be one of important molecular pathways for carcinogenesis and the molecular processes of HCC according to different hepatitis viruses are poorly understood. There are increasing evidences supporting the multistep process of hepatocarcinogenesis and dysplastic nodule (DN) is reported as a precancerous lesion. To evaluate epigenetic alteration during each step of hepatitis B virus related hepatocarcinogenesis, methylight studies for p16, APC, SOCS1, COX2 and RASSF1A were performed in 26 B viral chronic hepatitis (CH)/cirrhosis, 29 low grade DN, 14 high grade DN, 15 early HCC, and 29 advanced HCC. Methylation status of these 5 genes was increased according to progression of multistep hepatocarcinogenesis; hypermethylation status (>10%) of APC, RASSF1A and SOCS1 was found by 31% (8/26), 31% (8/26), and 15% (4/26) in CH/cirrhosis, respectively, and the hypermethylation status of these genes was 1.5~2 fold higher in DNs and HCCs compared to CH/cirrhosis ($p<0.05$). Similarly, gradual increase of methylation was found in p16 and COX2 ($p<0.05$). Among these 5 genes, the average numbers of methylated genes were 0.77, 1.79, 2.14, 2.73 and 2.21 for CH/cirrhosis, low grade DN, high grade DN, early HCC and advanced HCC, respectively, and this progressive increase from CH/cirrhosis to DNs through HCC may suggest that tumor suppressor gene methylation is an early event in hepatocarcinogenesis. We also examined methylation status of SPRY2, DCC and ERK of which epigenetic alteration is known to be important for tumorigenesis. These genes, however, did not show any DNA methylation in the various lesions of all cases. In conclusion, our data suggest that epigenetic alteration of p16, APC, SOCS1, COX2 and RASSF1A is an early event during HBV related hepatocarcinogenesis, which starts to occur in chronic B viral hepatitis/cirrhosis. Acceleration of methylation process is considered to be important for the progression of multistep hepatocarcinogenesis.

Cancer III (2168 – 2195)

2168/B637

Pericyte-like/ Mesenchymal Stem Cells Grown from Human Prostate Cancer Can Generate Atypical Glands under Appropriate Stimulation.

M. Crisan[1,2], D. Bacich[1,3], V. Yao[3], B. Sun[4,5], K. Dreher[3], A. Parwani[2], A. Logar[5], B. Peault[4,5,6], L. Croisille[1,2,6]; [1]Pathology, University of Pittsburgh Cancer Institute, Pittsburgh, PA, [2]Pathology, University of Pittsburgh , School of Medicine, Pittsburgh, PA, [3]Urology, Shadyside Hospital, Pittsburgh, PA, [4]Pediatrics, Children's Hospital, Pittsburgh, PA, [5]Stem Cell Research Center, Children's Hospital, Pittsburgh, PA, [6]McGowan Institute for Regenerative Medicine, Pittsburgh, PA

Background: The role of pericytes/perivascular cells in cancer biology is an important question, as these cells are possible targets for future therapies. Pericytes, in normal human tissues, can be isolated on CD146 expression, and absence of CD56, CD45 and CD34. Our aim is to isolate, characterize and amplify pericytes from human prostate tumors (CaP), to further assess their role. Methods: Cells were obtained from 7 CaPs specimens by 2 strategies: (a) after fluorescence-activated cell sorting based on CD146, CD56, CD34, CD45 markers or (b) after multiple steps of cell adhesion, and then seeded in vitro in pericyte culture conditions. After amplification, cells were characterized by immunocytochemistry, flow cytometry, RT-PCR, and subjected to in vivo assays in a xenochimeric model. Results: We obtained large numbers of cells displaying a pericyte-like morphology along both strategies. Pericyte markers (ALP, NG2, αSMA, CD146 and PDGFRβ) were expressed by 25 to 100% of the cells, while mesenchymal stem cell (MSC) markers (CD9, CD13, CD44, CD73 and CD90) as well as CD49b (alpha2 integrin) were expressed by 95% to 100% of the cells. Eight weeks after in vivo injection under the renal capsule of immunodeficient mice, in presence of fetal rat UGM, pericyte-like cells from 3 specimens (3/3) generated xenografts, while fetal rat UGM alone and pericyte-like cells alone didn't grow in vivo. The histologic analysis and immuno-labeling show the presence of glandular structures with prostate intraepithelial neoplasia (PIN) features, vessels and stroma, and at least some of the glands and some rare vessels are of human origin. Conclusions: Our results show for the first time that pericyte-like cells/MSCs can be obtained in large numbers from prostate tumors, and that these cells, under appropriate stimulation, are able to trigger the formation of, and/or differentiate into glandular structures displaying preneoplastic features. Further characterization of the respective origin (human, mouse, rat) of the glands, vessels, and stroma, is mandatory to precise the potential of these pericyte-like cells, that needs also to be compared to pericytes grown from normal prostates.

2169/B638

Toll-Like Receptors Response by Tumor and Epithelial Cells.

M. Shariati; Cell and Dev. Biology, University of California Irvine, Irvine, CA

The goal of this study was to compare the chemokine secretion in response to toll-like receptor (TLR) ligands, by two cell lines from two different organs, lung and breast with a view to understand organ specific immunity. We used a wide range of TLR ligands to determine interleukin 8 (IL-8) and interferon-inducible protein 10 (IP-10) secretion by the two cell lines lung epithelial (A549) and breast cancer (MCF-7). The hypothesis was that A549 would be more responsive to TLR ligands than MCF-7 due to the different environment that they reside in. Although cell culturing, freezing, and counting were involved in this experiment, we applied Enzyme-Linked ImmunoSorbent Assay (ELISA) as the main method to measure the secreted chemokines of our collected samples. The results indicated that TLRs on the breast

cancer cell lines respond weakly to the stimulators compared with lung epithelial cell lines. These results suggest that since lung is exposed to a number of infections compared to breast it is more able to respond to infections.

2170/B639

A Novel In Vitro Differentiation Model to Study Survival Signaling of Androgen Receptor Expressing Prostate Secretory Cells.

L. E. Lamb[1,2], B. S. Knudsen[3], C. Miranti[1]; [1]Laboratory of Integrin Signaling and Tumorigenesis, Van Andel Institute, Grand Rapids, MI, [2]Cell and Molecular Biology Program, Michigan State University, East Lansing, MI, [3]Division of Public Health Sciences, Fred Hutchinson Cancer Research Center, Seattle, WA

Background: Androgen receptor (AR) is critical for cell survival in the prostate gland. Adhesion of the non-AR expressing basal epithelial cells to extracellular matrix by integrins is required for their survival. However, the AR expressing secretory epithelial cells of the prostate are not adherent to matrix and the mechanisms by which they survive are poorly understood. Unfortunately, the ability to analyze secretory cells has been limited by our inability to culture these cells *in vitro*. Objective: Our objective was to develop an *in vitro* model for generating AR expressing prostate secretory cells. The cell signaling pathways that regulate survival of prostate secretory cells will be determined. Methods: Confluent primary prostate basal epithelial cells were induced to differentiate following treatment with KGF and androgen. After 14 days, differentiated AR-expressing secretory cells appeared as a secondary cell layer above the basal cells. Survival signaling pathways were identified using pharmacological inhibitors and TUNEL staining. Results: Following differentiation the bottom cell layer retains characteristics of the basal cell population; they express integrins, Bcl-2, and produce laminin (LM) 5 matrix. In contrast, the cells on the top layer express AR, secretory cell keratins K18 and K19, and several androgen-dependent markers including PSA, Nkx3.1, and KLK2. Also, they no longer express basal cell markers; Bcl-2, integrins or LM 5. Using pharmacological inhibitors we have demonstrated that in contrast to basal cells, secretory cells are not dependent on EGFR signaling for survival, but are dependent on PI-3K signaling for survival. Furthermore, androgen, the physiological ligand for AR, is unable to promote survival when PI-3K signaling is inhibited. Conclusion: We have generated an *in vitro* model of the prostate epithelium which recapitulates many *In Vivo* characteristics. Using this model, we have demonstrated that prostate secretory cells are dependent on PI-3K, but not EGFR, signaling for cell survival. This model will be a valuable tool for understanding the biology of prostate secretory epithelial cells, a cell population that was previously not available for analysis.

2172/B641

LOXL4 Alternative Splicing and Tumor Progression.

S. Sebban[1], B. Davidson[2], R. Reich[1]; [1]Pharmacology, Hebrew University, Jerusalem, Israel, [2]Pathology, Norwegian Radium Hospital, Oslo, Norway

Objective: The Lysyl oxidase-like 4 (LOXL4) protein has a catalytic domain which is similar to that of Lysyl Oxidase, but it is not well characterized otherwise. We have previously found LOXL4 to be alternativly spliced, in a site-specific pattern, in ovarian and breast carcinomas. In the present study, we over expressed LOXL4 and its splice variants in ovarian and breast cancer cell lines, and analyzed their metastatic potential. Methods: LOXL4 mRNA expression was analyzed in 125 ovarian carcinomas (70 effusions, 55 solid tumors), 79 breast carcinomas (21 effusions, 58 primary), using RT-PCR. Full length LOXL4 cDNA, splice variant 1 (SPLV1), and splice variant 2 (SPLV2) cDNAs, were cloned into the pcDNA3.1 expression vector (Invitrogen). Each isoform was transfected into the ES-2 ovarian cancer and the MDA435 breast cancer cell lines. Following cloning, the metastatic potential of each clone was assessed by invasion and attachment assays and by expression of various metastasis-related genes. Results: We identified two new splice variants of LOXL4. In ovarian effusions, SPLV1 was significantly elevated compared to solid lesions ($p<0.001$), and SPLV2 was expressed only in effusions. In breast carcinoma, solid tumors did not express LOXL4, while effusions expressed two of three variants. Analysis of the LOXL4 mRNA sequence for axon distribution revealed the spliced segments to be exon 9 (splice variant 1) or both exons 8 and 9 (splice variant 2). Within the encoded protein, exons 8 and 9 together are translated to a functional unit, and form SRCR4 (scavenger receptor cysteine rich 4). Each of the various LOXL4 variants was trasfected into ES-2 and MDA435 cell lines demonstrated a unique behavior. Cells over expressing the LOXL4 full length were similar in behavior to control cells, SPLV1 and SPLV2 expressing cells showed higher metastatic potential. Conclusions: Our data shows the importance of the alternative splicing of LOXL4 in tumor progression in ovarian and breast carcinoma.

2173/B642

Min Mice Exhibit Genomic and Phenotypic Instability That Precede LOH at Adenomatous Polyposis Coli.

K. Kaplan, C. M. Caldwell; MCB, UC Davis, Davis, CA

Familial adenomatous polyposis is associated with the inheritance of a single mutant allele of adenomatous polyposis coli (APC) and disease onset follows loss of heterozygosity at APC, leading to stabilization of β-catenin and cell proliferation. However, the most commonly occurring APC mutants act dominantly to cause mitotic errors and tetraploidy in the intestinal epithelium (Caldwell et. al., JCB, 2007), a state that is predicted to contribute to phenotypic instability. To characterize genomic and phenotypic instability in the gut and relate it to cancer progression, we examined genetic, epigenetic and cell fate markers in a series of different aged Min (APCMin/+) and wild type mice (prenatal to 20 weeks old). We observed no LOH or abnormal dysplasias in Min animals before 3.5 weeks of age even though mitotic errors and tetraploid cells were observed at a constant rate prior to the first signs of cancer progression. Intestinal cells from normal tissue in Min mice exhibited elevated DNA damage and decreased acetylation of histone H4 compared to wild type animals. Significantly, we found that crypts in Min animals have elevated levels and altered patterns of the stem cell marker, Mushashi. This finding suggests that the genetic and epigenetic instability in Min animals affects cell fate prior to cancer progression. Similar changes were also observed in the earliest cancer lesions and persisted in dysplasias found in mice older than 14 weeks. Furthermore, we observed that in some cancer lesions, stabilization of β-catenin occurs independent of the loss of APC, thus raising the possibility that early instability generates multiple paths for cancer progression. The observations that APC dominant mutations contribute to genetic and epigenetic instability prior to cancer progression and alters stem cell fate leads us to propose that such instability may be a critical characteristic of cancer stem cells.

2174/B643

The Human Y-Encoded Testis Specific Protein (TSPY) Interacts with a Putative Oncoprotein EEF1A and Enhances Gene Expression.

T. Kido, Y. Lau; University of California, San Francisco, San Francisco, CA

The TSPY gene was initially identified as a testis specific gene tandemly repeated at the critical region of the gonadoblastoma locus on the Y-chromosome (GBY). GBY harbors the gene(s) capable of predisposing the dysgenetic gonads of sex-reversed females and intersex patients to develop gonadoblastoma. TSPY is normally expressed in testicular germ cells, and abundantly expressed in gonadoblastoma and various types of testicular germ cell tumors, including carcinoma-in-situ and seminoma. In addition to germ cell tumors, it is also frequently expressed in somatic cancers including hepatocellular carcinoma and prostate cancer. Although numerous evidences support the hypothesis that TSPY is involved in tumorigeneis, the exact role of TSPY is currently unknown. TSPY is a member of TSPY/SET/NAP1 superfamily, whose members harbor the highly conserved NAP-domain that mediates protein-protein interactions. To explore the possible mechanism of TSPY in tumorigenesis, we had performed a yeast two-hybrid screen of a fetal gonadal cDNA library using TSPY NAP-domain as bait, and identified the translation elongation factor EEF1A as a binding partner of TSPY. EEF1A is essential for translation elongation during protein synthesis, and its expression level is correlated with cell viability and proliferation. Recent studies showed that EEF1A could potentially be involved in various types of cancers, including prostate, ovarian and breast cancers. Immunohistochemical analysis demonstrated that TSPY and EEF1A were highly expressed and colocalized in tumor germ cells of human seminoma specimens, suggesting their possible interaction in tumor cells. TSPY was capable of enhancing protein synthesis of reporter gene in the transfected culture cells. Such TSPY-enhanced protein synthesis was augmented by an overexpression of EEF1A. TSPY also increased the nuclear redistribution of eEF1A, resulting a parallel increase in reporter gene transcript. Since cell proliferation requires adequate protein synthesis to support the metabolic and growth requirements, TSPY could exert its oncogenic function by interacting with eEF1A and stimulating gene expression via its enhancement in protein synthesis and gene transcription.

2175/B644

Over-Expression of Zip6 (LIV1) in Tumorigenic Breast Cancer Cells (T47D) Cells Modulates Sub-Lethal Cytochrome C Release and Apopotgenisis.

V. Lopez, S. Kelleher; Penn State, University Park, PA

Breast cancer is one of the leading causes of death among women. The etiology is unknown; however, compelling evidence implicates dysregulation of zinc (Zn) homeostasis in breast cancer cells. Humans studies have suggested that over-expression of the zinc importer Zip6 (LIV-1), may be a useful prognostic marker for breast cancer. However, the functional relevance of increased Zip6 expression is not understood. We hypothesize that increased Zip6 expression dysregulates intracellular Zn pools affecting discrete cellular functions, such as apoptosis. Using quantitative real-time PCR, we determined that Zip6 mRNA level was 2-fold lower in tumorigenic breast cells (T47D) compared to normal human breast cells (HME). Conversely, Zip6 protein abundance was significantly higher compared with normal breast cells. Moreover, while Zip6 resided exclusively on the plasma membrane in HMEs reflecting its role as a Zn import protein, ~90% of Zip6 was located intracellularly in T47D cells. To explore the functional role of Zip6 in tumorigenic breast cells, we transfected T47D cells with Zip6 siRNA and examined cytoplasmic Zn levels, vesicular Zn pools and apoptogenesis. Co-transfection of a metal-responsive element (MRE)-luciferase reporter construct in Zip6 attenuated cells significantly reduced cytoplasmic Zn pools in T47D cells. Additionally, Zip6 attenuation significantly reduced vesicular Zn pools as detected with FluorZin-3 AM. To examine affects of diminished cellular Zn pools on cellular function, Zip6 attenuation significantly decreased the sub-lethal release of cytochrome C and subsequently blocked apoptosis by inhibiting caspase-3 and -9 activities. Taken together, this suggests that the tumorigenic potential of T47D cells could be mediated by alterations in intracellular Zn pools via Zip6.

2176/B645

Ceramide Induces Overexpression of Gene of Glycosylceramide Synthase in Tumour Cells Derived from Human Cervix-Uterine Cancer Infected with HPV Type 18 but It Does Not in Cells Non-infected with papillomavirus.

M. Gomez[1], A. Parra[1], A. Miliar[1], R. Lopez[2], A. Salas[1], G. Gutierrez[1]; [1]Posgrado, Medicine Highschool, Mexico City, Mexico, [2]Cell Biology, National Institute of Cardiology, Mexico City, Mexico

Ceramide is a potentially important mediator of several agents that affect cell growth, viability, and apoptotic and no apoptotic death. This lipid is derived from sphingomyelin hydrolysis, or produced by the de novo synthesis. We previously have demonstrated that ceramide inhibits the proliferation and induces death like necrosis of cervix-uterine cancer cells (CaCu) infected with papillomavirus type 18 (HPV-18), but it does not inhibit the proliferation of normal cells or cells form CaCu non infected. The resistance to the toxic effect of ceramide could be asociated with a expression of a molecule that glycosylate ceramide called glucosylceramide synthase (GCS). Due ceramide is glycosylated by this synthase, the toxic effect of ceramide is lost. In this work, we evaluated the possible differences in the expression of the gene of glycoylceramide synthase in tumoral cell lines infected with papillomavirus type 18 (cell line Caski) and tumoral cells non infected (cell line ViBo). We analyzed by RT and real-time PCR the expression of glucosylceramide synthase gene (gene bank: UGCG). The results shown that unexpected, CasKi cells treated with ceramide presented an overexpression of GCS over 40 times more with respect to the control (untreated cells), and ViBo cells diminished the expression. Its evident that ceramide induces the overexpression of GCS but cells finally and these events not occur in non-infected cells which are resistant to the toxic effect of ceramide. We propose that others mechanism are involved like a posttranscriptional changes in the gene of GCS or possibly participate other molecules related to multidrug resistance.

2177/B646

HEF1, Not P130 CAS Is Over-Expressed in Invasive Breast Ductal Carcinomas.

E. Pugacheva, S. McLaughlin, R. Cline; MBR Cancer Center, West Virginia University, Morgantown, WV

HEF1 and p130Cas are highly homologous adhesion scaffolding proteins involved in integrin-dependent signal transduction cascade. Both proteins universally expressed and share substantial similarities in migration associated functions. Previous work has indicated that over-

expression of p130Cas correlates with poor breast cancer patient outcome and recurrences. Reagents available for study of p130Cas are in many cases incapable to distinguish between HEF1 and p130Cas due to high homology between these proteins. Nevertheless only HEF1was found to be important for activation of mitotic kinase AurA, which is constitutively active in many types of cancers, including 60% of breast cancers. There are no comprehensive data about HEF1 expression to compare with p130Cas in breast cancers. To fill this gap, and support our previous work on breast cancer cell lines, we developed an HEF1 specific monoclonal antibody and selected a p130Cas antibody, which does not cross react with HEF1. We then tested antibody on a set of normal and transform breast cell lines and paraffin embedded tissue sections of normal versus breast carcinoma samples. Intriguingly, we found that breast invasive ductal carcinoma (HCC1937) and breast adenocarcinoma (MDA-MB468, MCF-7, HCC1428) cells differentially express HEF1 protein, but not p130Cas to compare with non tumorogenic breast epithelial cells (MCF10A or MCF12F). Breast ductal carcinoma lysates demonstrate HEF1 over-expression only in samples with advanced stages of disease: invasive and metastatic. To assess whether HEF1 protein is over-expressed in breast cancers we arranged collaborative study with Yale Tissue Microarray Facility and screened TMA (Tissue Micro-Array) representing 43 different breast cancer cases. The TMA was analyzed for HEF1 protein expression by fluorescent immunohistochemistry (IHC). About 70% of cases demonstrated visual increase in HEF1 protein level. HEF1 over-expression separated from p130Cas has not been previously reported for breast cancers, and may represent a class of breast carcinoma cases that have an unappreciated increase in invasive and proliferative potential.

2178/B647

Distribution of Interleukin-19 in Healthy and Neoplastic Tissue.

C. Hsing[1,2], J. Shieh[1,2], H. Li[3], C. Chu[1,2], M. Chang[1,4]; [1]Medical Research, Chi-Mei Medical Center, Tainan, Taiwan, [2]Anesthesiology, Chi-Mei medical center, Tainan, Taiwan, [3]Basic Medical Science, National Cheng Kung University, Tainan, Taiwan, [4]Biochemistry and Molecular Biology, National Cheng Kung University, Tainan, Taiwan

The influence of interleukin (IL)-19, a recently discovered cytokine in the IL-10 family, on tissue is still unclear. Our aim was to determine the distribution of IL-19 expression and to delineate the cell types that express IL-19 in healthy and neoplastic tissue, because this information will significantly facilitate the exploration of its pathophysiological functions. We used tissue microarray technology and an immunohistochemical survey with an anti-IL-19 monoclonal antibody to examine the expression of IL-19 in 28 healthy and 15 neoplastic tissues. IL-19 protein was positively stained in 15 healthy tissue-types and three major cell-types: epithelial cells, endothelial cells, and macrophages. We also found that several types of tumor cells were positively stained for IL-19, especially in squamous cell carcinoma (SCC) of the skin, tongue, esophagus, and lung, invasive duct carcinoma of breast, and transitional cell carcinoma of bladder. Using RT-PCR, we determined that SCC tissues of the oral cavity expressed mRNA of IL-19 and its receptors. In two cancer cell lines, OEC-M1 and OC-3, derived from SCC of oral cavity tumor tissues, IL-19 specifically activated an intracellular signal AKT. IL-19 also induced proliferation of the cells, which indicated that IL-19 may play as an important role in tumor biology and act in an autocrine manner in SCC tumors. This study provides important references for further investigation of the biological functions and clinical implications of IL-19 in humans.

2179/B648

Keratin 17 Impacts "Chronic Hyperproliferative Disease" by Modulating Inflammation.

D. J. DePianto, M. L. Kerns, P. Coulombe; Biological Chemistry, Johns Hopkins University, Baltimore, MD

Keratins, members of the intermediate filament gene family, exhibit tightly regulated spatiotemporal expression patterns within epithelial tissues. Intermediate filament networks provide mechanical support upon incident trauma or strain as evidenced by epidermolysis bullosa symplex, a skin blistering disorder in which either keratins 5 or 14 are mutated. Recently, there is evidence that intermediate filaments also play an active role in basic cellular processes such as protein translation and cell survival, polarity, and migration. Carcinomas typically feature changes in the composition or amplitude of keratin expression. Little is known, however, about the role of IF proteins within the context of cancer, and their potential contribution to the initiation, progression, and maintenance of tumorigenesis. Basal cell carcinoma (BCC) is a common form of skin cancer. Keratin 17 (K17), normally restricted to epithelial appendages in normal skin, is consistently upregulated within BCC tumor cells. We have investigated the impact of K17 loss on tumor formation and dynamics in an established transgenic mouse model of BCC. We uncovered a gender bias in the formation of ear neoplasias, whereby female mice show a delay in the age of tumor onset. Male transgenic mice develop ear neoplasia at postnatal day 72 (average),while females do so at day 84. Absence of K17 significantly impacts ear tumors, delaying the average age of onset to 89.5 and 101.9 for males and females, respectively. This delay is accompanied by a 3.5 fold decrease in mitotic activity in transgenic mice lacking K17 protein, but no change in apoptosis or size of individual tumor cells. The gender-based, and K17 status-based, differences in tumor onset each closely correlate with the extent of neutrophil infiltration. Studies of a distinct, tumor-free, acute paradigm of mouse skin inflammation revealed that loss of K17 is again associated with reduced neutrophil infiltration. Altogether these findings point to a novel role for K17 in modulating the propagation of inflammatory signals within skin epithelia, an intriguing prospect given the close association of this keratin with wound repair, psoriasis, viral infections, and cancer. Supported by NIAMS and NCI.

2180/B649

Caveolin-1-Mediated Suppression of Cyclooxygenase-2 Expression via a β-Catenin-Tcf/Lef-Dependent Transcriptional Mechanism Reduced PGE2 Liberation and Survivin Expression.

D. Rodriguez[1], J. Tapia[2,1], J. G. Fernandez[1], V. Torres[1], N. Muñoz[1], D. Galleguillos[1], L. Leyton[2,1], A. F. Quest[1,2]; [1]Centro FONDAP de Estudios Moleculares de la Célula (CEMC), Facultad de Medicina, Universidad de Chile, Santiago, Chile, [2]Programa de Biologia Celular y Molecular, ICBM, Facultad de Medicina, Universidad de Chile, Santiago, Chile

OBJECTIVE: Enhanced cyclooxygenase-2 (COX-2) as well as increased production and secretion of prostaglandin E2 (PGE2) are associated in human cancer cells with increased proliferation and resistance to apoptosis. On the other hand, Caveolin-1 (Cav-1), a structural protein required for the formation of caveolae, functions as a tumor suppressor in some cancer cells. The mechanisms explaining this ability of Cav-1

remain controversial. In particular, the possibility that caveolin-1 regulates the expression of COX-2 has not been explored. METHODS: Cells were transiently or stably transfected with placIOP(Cav-1). Reporter assays and semi-quantitative PCR were employed to assess transcriptional changes and Western blotting to evaluate changes at the protein level. Protein localization was determined by confocal microscopy and multi-protein complex formation by co-immunoprecipitation. PGE2 levels were measured using a commercially available kit. RESULTS: Caveolin-1 re-expression in cells lacking this protein, such as human colon cancer (HT29, DLD-1), breast cancer (ZR75) and embryonic kidney (HEK293T) cells reduced COX-2 mRNA and protein levels, β-catenin-Tcf/Lef and COX-2 gene reporter activity, as well as the production of PGE2 and cell proliferation. Moreover, COX-2 overexpression or PGE2 supplementation increased levels of the inhibitor of apoptosis protein survivin by a transcriptional mechanism, as determined by PCR analysis, survivin gene reporter assays and Western blotting. Furthermore, addition of PGE2 to the medium prevented effects attributed to caveolin-1-mediated inhibition of β-catenin-Tcf/Lef-dependent transcription. Finally, PGE2 reduced the co-immunoprecipitation of caveolin-1 with β-catenin and their co-localization at the plasma membrane. CONCLUSIONS: Caveolin-1 reduced COX-2 and survivin expression, as well as liberation of PGE2 via a β-catenin/Tcf-Lef-dependent transcriptional mechanism. Furthermore, PGE2 supplementation overcame Cav-1-mediated limitations by dispersing a plasma membraane-bound, multiprotein complex containing Cav-1/β-catenin. Acknowledgements: FONDAP15010006 (AFGQ), FONDECYT1070699 (LL), FONDECYT3050037 (JT), Mecesup (DR) and CONICYT (GF).

2181/B650

Tumor Cells Overexpress ISG15 through Type I Interferon Signaling.

Y. Tsai[1,2], S. Pestka[3,2], L. F. Liu[1,2]; [1]Pharmacology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ, [2]Graduate School of Biomedical Sciences, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ, [3]Molecular Genetics, Microbiology and Immunology, UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ

Our previous studies have shown that ISG15, the interferon (IFN)-stimulated gene 15, is highly overexpressed in many human tumor tissues and tumor cell lines. ISG15 is known to mediate innate immune response and inhibit viral production. However, its role in tumorigenesis is largely unknown. Here we want to identify upstream factors for ISG15 overexpression using cell culture models. Using breast cancer ZR-75-1 cells (known to overexpress ISG15), we have shown that ISG15 expression is significantly reduced by the addition of the neutralizing antibody against IFN-β but not IFN-α (both are type I IFN). The presence of IFN-β in tissue culture medium was also suggested from an experiment using conditioned medium harvested from ZR-75-1 to stimulate ISG15 expression in breast cancer T47D cells (known to express a low level of ISG15), as well as direct determination of its concentration using an antiviral assay. Furthermore, the involvement of interferon (alpha, beta and omega) receptor 1 (IFNAR1) in regulating ISG15 expression was demonstrated by siRNA knockdown experiments. Together, these results suggest that ISG15 overexpression in breast cancer ZR-75-1 cells is due to elevated IFN signaling. We also determined the roles of oncogenes in regulating ISG15 expression. ISG15 was shown to be up-regulated in NIH-3T3 cells transiently transfected with Harvey rat sarcoma viral oncogene (v-Ras), as well as in NIH-3T3 cells transformed with v-Ras. In addition, ISG15 was shown to be elevated by viral sarcoma oncogene (v-Src) since reduced ISG15 expression was observed in both wild-type rat intestinal epithelial (RIE) cells and temperature sensitive v-Src transformed RIE cells at the non-permissive temperature. In conclusion, these results suggest that ISG15 is up-regulated by both the type I IFN signaling pathway and oncogenes in tumor cells. Further study is necessary to understand how IFN signaling affects cancer cell phenomena such as apoptosis, metastasis and tumor immunoediting.

2182/B651

Abrogation of Nucleolar Localization of Tumor Suppressor Function in Human Breast Cells Is Sufficient For rRNA Upregulation and Transformation.

S. Rossetti[1], J. Esposito[1], A. T. Hoogeveen[2], N. Sacchi[1]; [1]Cancer Genetics Program, Roswell Park Cancer Institute, Buffalo, NY, [2]Clinical Genetics, Erasmus MC, Rotterdam, Netherlands

The nucleolus, the site of production and assembly of ribosomes, is also the site of of tumor suppressor and oncogene activities. Previously, we described that one oncoprotein of the Myeloid Translocation Gene (MTG) family of proteins, MTG16a, is endowed with a novel region essential for nucleolar targeting. Here we show that loss-of-MTG16a function in the nucleolus is sufficient to induce tumorigenesis. The relocalization of MTG16a from the nucleolus into the nucleoplasm of untransformed breast epithelial cells can fully recapitulate the effects of MTG16a knock down, which include the appearance of a transformed morphological phenotype as well as the increase of argyrophilic nucleolar organization regions. Consistently, MTG16a loss/relocalization leads to upregulation of pre-rRNA transcription. This study provides the proof of principle that MTG16a performs its tumor suppressor function in the nucleolus, likely by keeping in check rRNA transcription. This work was supported, in part, by the NCI Cancer Center Support Grant to Roswell Park Cancer Institute (CA016056), and a postdoctoral fellowship of the Susan Komen Foundation (SR).

2183/B652

Human Papillomavirus16 E5 Initiates Cell Transformation through Cell-Cell Fusion.

L. Hu[1], P. C. Angeletti[2], B. Ceresa[1]; [1]Cell Biology, University of Oklahoma Health Sciences Center, Oklahoma City, OK, [2]Nebraska Center for Virology, University of Nebraska, Lincoln, NE

The Human papillomavirus (HPV) is a non-enveloped DNA virus. Infection with certain types of HPV, such as HPV16, is associated with a number of anogenital cancers and cancers of the head and neck. The molecular mechanism of HPV16 induced carcinogenesis is not fully understood. Of the 8 genes that are encoded by the HPV16 genome, three are regarded as oncogenic based on their ability to transform cells - E5, E6, and E7. The E6 and E7 oncogenes have well-established roles in promoting tumor propagation by blocking cell cycle checkpoint inhibitors (p53 and Rb). The role of E5 has been controversial. We recently discovered that when cells are transfected with the HPV16 genome they give rise to bi-nucleated cells; transfection with the HPV16 genome with a functional deletion of the E5 gene does not. Expression of HPV16 E5 alone gives rise to bi-nucleated cells. This is noteworthy since bi-nucleated cells are characteristic of pre-cancerous lesions. Using live cell video microscopy and quantitative biochemical assays, we have found that HPV16 E5 gives rise to bi-nucleated cells through cell-cell fusion. The functional consequence of cell-cell fusion is reflected in the growth properties of these cells. Most bi-nucleated

cells fail to propagate. However, when bi-nucleated cells are engineered to express HPV16 E6/E7 to suppress cell cycle checkpoint inhibitors, there is an increased likelihood that cells can escape growth arrest and become transformed as measured by colony formation. This is the first report that HPV16 E5 is necessary and sufficient for the formation of bi-nucleated cells through cell-cell fusion. Our findings along with clinical data provide the groundwork for a model of the coordinated activity of the three HPV16 oncogenes leading to cell transformation. This model is supported by our in vitro experiments.

2184/B653

Pediatric Tumor Cell Lines Contain a Population of Putative Stem Cells and Express Early Neural and Glial Progenitor Markers.

J. Ozolek[1], J. E. Esplen[1], V. S. Donnenberg[2], A. D. Donnenberg[2]; [1]Pathology, Children's Hospital of Pittsburgh, Pittsburgh, PA, [2]University of Pittsburgh Cancer Institute, Pittsburgh, PA

Objectives: The cancer stem cell may be the optimal therapeutic target for many tumors and putative stem cells have been isolated from several adult tumors. Pediatric tumors such as pancreatoblastoma (PB), Wilms tumor (WT) and hepatoblastoma show evidence of multi-lineage differentiation. The aim was to identify and characterize a population of putative stem cells from cell lines derived from pancreatoblastoma, Wilms tumor and hepatoblastoma. Methods: Cell lines from two PB, two WT, and hepatoblastoma were established. One PB line (CMBS) was generously provided by Dr. Carol Thiele at the NCI and the hepatoblastoma (HUH-6) was acquired from the Health Sciences Research Bank from Japan. Another PB line (PBJ) cells grew better in standard media with gelatin and bFGF (PBJ+). Flow cytometry was performed on CMBS, PBJ+, and PBJ grown without bFGF (PBJ-) for markers CD44, CD90, CD117, CD133, and cytokeratin using a 3-laser, 9-color Dako CyAn LX cytometer. Immunocytochemistry was performed by standard methods for neuroglial markers. Results: Flow cytometry was available for the PB and Wilms1 lines. PBJ+ and PBJ- had high coexpression of CD44 and CD90 (84.68% and 90.35% respectively) while CMBS had only 0.14%. Forty-percent of CMBS had coexpression of CD44 and CD117 while PBJ lines had very low expression of CD117. CD133 expression was low in all PB lines. Wilms1 line expressed CD44, CD90, CD133, and cytokeratin with very low CD117 expression. PB and WT lines had diffuse expression of vimentin and PDX-1. Low level Oct4 expression was present in the CMBS line and in later passage Wilms1. PBJ, CMBS, Wilms1, and Wilms2 had widespread expression of nestin, doublecortin, olig2, SOX2, and 3CB2. These tumors also had expression of astrocytic and mature neural lineage markers (S100β, GFAP, Class III beta-tubulin). HUH-6 expressed SOX2 with little to no expression of other neuroglial markers. Conclusions: PB and WT cell lines show divergent stem cell phenotypes by flow cytometry and widespread early neuroglial marker expression. Hepatoblastoma shows a more restricted and undifferentiated phenotype. Further investigation of pediatric tumors for cancer stem cells will be crucial to identify new therapeutic targets.

2185/B654

Furan Toxicity and Effects on Cell Cycle in Rat and Human Hepatoma Cells In Vitro.

T. J. Schrader, I. Langlois; Toxicology Research Division, Health Canada, Ottawa, ON, Canada

Objectives: Furan arises in cooked or heated foods through reactions of amino acids, fatty acids, and ascorbic acid. Furan is carcinogenic in rodent models and has been classified as group 2B, possibly carcinogenic to humans, by the International Agency for Research on Cancer (IARC). In order to explore the carcinogenic mechanism involved and possible differences in species sensitivities, furan toxicity and cell cycle effects were compared in rat H4IIE and human HepG2 hepatoma cell lines. Methods: Cells were plated into 96 multiwell plates (300 ul/well) and allowed to attach overnight. Due to volatility, chemical dilutions and additions were made on ice and the plates sealed with plastic film before further incubation. Cell viability, after 24 and 48 hours of incubation, was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay. Cells with or without low serum synchronization were analyzed for cell cycle effects by flow cytometry (Guava Easycyte) with propidium iodide staining (Guava Cell Cycle Reagent) according to manufacturers instructions. Results: H4IIE and HepG2cells exhibited somewhat different sensitivities to furan with LD50's of 1.8 and 0.96 µl furan/well respectively. With nonsynchronized cells, cell cycle effects were only observed at levels of furan showing toxicity, with the proportion of cells in G0/G1 decreased while S-phase cells were increased. A small initial increase in G2/M cells at 1 µl furan/well was followed by G2/M cell loss after treatment with 2-3 µl/well. Synchronized HepG2 cells also showed increased S and decreased G2/M populations beginning at 0.75 µl/well. However, furan decreased both S and G2/M populations in synchronized H4IIE cells beginning at 0.75 µl/well. Conclusions: The increased sensitivity of human HepG2 cells to furan suggests that increased care is needed in developing health risk assessments. The increased proportion of S-phase cells found in both lines, at least under nonsynchronized conditions, may have implications for carcinogenesis.

2186/B655

Structural and Functional Studies of Stanniocalcin-1 (Stc1), a Potential Microenvironment Marker for Childhood Acute Lymphoblastic Leukemia (ALL).

D. M. Trindade[1,2], M. T. Santos[1,2], J. A. Yunes[3], J. Kobarg[1,2]; [1]Molecular Biology Laboratory (LBM), Brazilian Synchrotron Light Laboratory (LNLS), Campinas, Brazil, [2]Biochemstry and Genetic, University of Campinas (Unicamp), Campinas, Brazil, [3]Centro Infantil Boldrini, Campinas, Brazil

Leukemia cells interact with the bone marrow (BM) microenvironment, which provides them proliferative advantages. STC1 is widely expressed in various tissues and is shown to be present in several types of cancer as well as during angiogenesis but neither its receptor nor its exact functions are known in mammals. The aim of our work is to study the molecular properties and partners of STC1 for better understanding its role within the BM microenvironment. We stimulated BM stromal cells by direct contact with primary ALL cells or by the addition of plasma from ALL patients to the culture medium and then performed microarray analysis of gene expression of the stromal cells. Using quantitative RT-PCR analysis we showed that STC1 is among the genes that showed higher levels of activation in BM stromal cells by leukemia cells vs. controls. STC1 cDNA was amplified and cloned into vectors for heterologous protein expression and yeast two-hybrid assays. Full-length STC1 did not fold well (insoluble expression) in bacteria however its carboxy-terminal half showed some soluble expression when fused to GST. Based on co-expression of GFP with STC1 by using a bi-cistronic vector in the baculovirus system a new protocol for cell culturing and highly efficient production of secreted STC1 was obtained. In parallel, yeast two-hybrid (Y2H) screening of

human cDNA libraries were conducted in order to identify STC1 interacting proteins. The proteins found corresponded well to several functional and localization data reported so far for STC1, including mitochondria, endoplasmic reticulum, nucleus, and putative transmembrane receptors. Ongoing pull-down experiments are being performed to confirm these interactions. Furthermore crystallization trials and spectroscopic assays are ongoing. Financial support: FAPESP, CNPq and LNLS.

2187/B656

Id1 Overexpression Induces Tetraploidization and Multiple Abnormal Mitotic Phenotypes by Modulating Aurora A.

C. Man[1], s. doxsey[2], S. Tsao[1]; [1]Anatomy, The University of Hong Kong, Pokfulam, Hong Kong, China, [2]Molecular Medicine, University of Massachusetts Medical School, Worcester, MA

The basic helix-loop-helix transcription factor, Id1, was shown to induce tetraploidy in telomerase-immortalized nasopharyngeal epithelial cells in this study. Using both transient and stable Id1-expressing cell models, multiple mitotic aberrations were detected; including centrosome amplification, binucleation, spindle defects and microtubule perturbation. Many of these abnormal phenotypes have previously been reported in cells overexpressing Aurora A. Further experiments showed that Id1 could stabilize Aurora A, while knocking down Aurora A expression in Id1-expressing cells could rescue some of the mitotic defects. The mechanisms by which Aurora A could be modulated by Id1 were explored. DNA amplification of the Aurora A locus was not involved. Id1 could only weakly activate the transcriptional activity of the Aurora A promoter. We found that Id1 overexpression could affect Aurora A degradation, leading to its stabilization. Aurora A is normally degraded from mitosis exit by the APC/C Cdh1-mediated proteasomal proteolysis pathway. Our results revealed that Id1 and Cdh1 are binding partners. The association of Id1 and Cdh1 was found to be dependent on the canonical destruction box (D box) motif of Id1; the increased binding of which may compete with the interaction between cdh1 and Aurora A, leading to stabilization of Aurora A in Id1-overexpressing cells.

2188/B657

Chip-Chip in Primary AML Blasts Identifies Peroxiredoxin-II as an Epigenetically Silenced Tumor Suppressor.

S. Agrawal-Singh[1], M. Wiechmann[1], C. Nolte[1], N. Thoennissen[1], H. Klein[2], M. Dugas[2], W. E. Berdel[1], S. Koschmieder[1], H. Serve[3], C. Müller-Tidow[1]; [1]Hematology and Oncology, Muenster, Germany, [2]Medical Informatics and Biomathematics, Muenster, Germany, [3]Hematology and Oncology, Frankfurt, Germany

Epigenetic silencing of tumor suppressors is a frequent event in leukemogenesis. We performed ChIP-Chip to identify genes with altered Histone H3 acetylation in primary AML specimens (n=115) and normal CD34+ progenitor cells (n=21). Interestingly, promoters of several members of the peroxiredoxin family (PrdxII and Prdx IV) were identified to be significantly Histone H3 hypoacetylated in primary AML blasts. Peroxiredoxins (Prdx) are antioxidant enzymes that regulate the amount of reactive oxygen species in the cell. Prdx scavenge H2O2 and protect cells from oxidative damage to cellular DNA, lipid and proteins. Confirmation by quantitative PCR revealed that the PrdxII promoter was hypoacetylated in AML, and mRNA expression was 10-fold induced in U937 cells upon exposure to the demethylating agent 5-Azadeoxycytidine (Aza). Demethylation of U937 cells by Aza resulted in increased Histone H3 acetylation at the PrdxII promoter. DNA hypermethylation was frequent in primary AML blasts (18/103) but not in control samples (0/40) as assessed by methylation-specific PCR. Hypoacetylation of Histone H3 and DNA hypermethylation was associated with repression of PrdxII mRNA and protein levels in primary AML samples. Decreased PrdxII protein expression as assessed by immunohistochemistry in a tissue microarray was associated with a poor prognosis in AML patients. On the functional level, PrdxII inhibited the growth of hematopoietic progenitor cells in colony assays and negatively influenced receptor tyrosine kinase signaling by inhibition of ERK and AKT activation upon overexpression. Conversely, PrdxII knock-down by shRNA in myeloid progenitor cell lines and in murine primary bone marrow cells led to enhanced growth and tyrosine kinase signaling. A decrease in PrdxII levels was associated with an increase in reactive oxygen species and enhanced phosphorylation of STAT5, AKT and ERK. Taken together, these findings suggest that chromatin modifications in AML suppress expression of PrdxII. Our data also revealed that PrdxII is a novel putative tumor suppressor in AML.

2190/B659

Role of Transient Receptor Potential Vanilloid Type-2 (TRPV2) in Cell Proliferation and Chemo-resistance of Human U87 Glioma Cells.

M. Morelli[1, 2], C. Amantini[1], L. Ricci-Vitiani[3], R. Pallini[4], A. Arcella[5], S. Caprodossi[1], F. Giangaspero[2, 5], R. De Maria[3], S. Pimponi[1], G. Santoni[1], M. Nabissi[1]; [1]Experimental Medicine and Pubblic Health, University of Camerino, Camerino, Italy, [2]Department of Experimental Medicine, University of Rome "Sapienza", Rome, Italy, [3]Istituto Superiore di Sanità, Rome, Italy, [4]Department of Neurosurgery, Catholic University, Rome, Italy, [5]I.R.C.C.S. I.N.M. Neuromed, Pozzilli, Italy

Two marked features of glioblastoma multiforme (GBM) aggressiveness, that contributed to the poor prognosis associated with these tumors, are the resistance to chemotherapeutic drugs and the high tumor proliferation rate. Aim of this study was to investigate the expression of the transient receptor potential vanilloid-2 (TRPV2) in 64 cases of gliomas with histological grade ranging from I to IV (WHO classification), in order to identify a relationship between changes in TRPV2 expression and malignant progression. TRPV2 expression, evaluated by duplex-qRTPCR, immunohystochemistry and western blot analyses, was found inversely correlated with grading ($p < 0.01$). The results indicated that decrease of TRPV2 gene expression was associate with malignant progression. To investigate the functional significance of TRPV2 in human GBM, small interfering RNA was used to silence the expression of TRPV2 gene in U87MG cells. qRTPCR and immunoblotting analyses showed that TRPV2-siRNA potently inhibited the TRPV2 mRNA and protein expression. TRPV2-siRNA U87MG cells displayed a marked down-regulation of CD95/Fas and TGF1β gene expression associated with a significant up-regulation of CCNE-1, CDK2, E2F1 and Raf-1 genes, as evaluated by RT-Profile PCR array. MTT assay and cytofluorimetric analyses showed that TRPV2-siRNA U87MG cells were significantly more resistant to Etoposide and BCNU treatment and displayed a significant decrease in Annexin-V staining. TRPV2-siRNA U87 MG cells also showed an increased number of BrdU positive cells, an increased cell proliferation and ERK phosphorylation level as compared to controls. Finally, by cytofluorimetric analysis the effect of TRPV2 gene silencing, on differentiation-related genes in U87MG cells have been also evaluated. TRPV2-siRNA reduced the GFAP+ and increased βIII-tubulin+ percentage of U87MG cells. Overall, these

findings revealed a novel role for TRPV2 in the control of glioma cell proliferation, differentiation and cell death. The involvement of TRPV2 in mediating resistance to DNA-damaging agent therapy may have important implications for improving the targeting and treatment for drug-refractory glioblastomas.

2191/B660

Human Epidermal Growth Factor Receptor-2(HER-2/Neu) Amplicon Size Variation in Breast Cancer Patients and Its Correlation with Prognosis.

W. Lim[1], S. Ahn[1], W. Han[2]; [1]University of Ulsan College of Medicine and Asan Medical Center, Seoul, South Korea, [2]Seoul National University Hospital, Seoul, South Korea

Objective: The purpose of this study is to examine HER-2/neu amplicon in the tissue of breast cancer paients and whether the variable amplicon size may be associated with prognosis. Methods: Amplification mapping by fluorescence in situ hybridization (FISH) and Reverse transcription PCR (RT-PCR) was performed in tissue of 50 breast cancer patients underwent surgery between 1999 and 2001. We analyzed the result of HER-2 amplicon by RT-PCR with clinical data including survival. Results : Twenty four of 50 patients had ERBB2 amplification and amplification mapping by fluorescence in situ hybridization and RT-PCR revealed a common region of amplification containing 6 highly expressed genes associated with ERBB2 amplification (MLLT6, PIP5K2B, LASP1, PPARBP, THRAP4, WIRE) and HER-2/neu amplicon size variation was showed also in human breast cancer as in cancer cell lines. There was no amplification of genes located far from ERBB2 when genes near ERBB2 was not amplified. The amplicon size increased in proportion to level of ERBB2 amplification. In ERBB2 amplified patients, co-amplification of PRARBP was related with worse disease-free survival but there was no statistical significance due to small number of patients. Conclusions: Among Her-2/neu overexpressed patients, those with different prognosis will be able to be selected by the analysis of variable amplicon size and in the future, detection of co-amplified gene associated with aggressiveness or suppression of tumor will be used in molecular target therapy.

2192/B661

MUC1, MUC2 and MUC5AC Expression in Hepatocellular Carcinoma with Cardiac Metastasis: Biomarkers for Malignancy?

P. Chu; Medicine, Chang Gung Memorial Hospital and University, Taipei, Taiwan

Background: The prognosis of advanced hepatocellular carcinoma is poor; it is even worse when cardiac metastasis is present. Because many recent studies indicate that mucin expression is important in cancer metastasis and recurrence, we investigated mucin expression in hepatocellular carcinoma patients with cardiac metastasis compared with primary hepatocellular carcinoma. Methods: Over a 6 year period, expression patterns for MUC1, MUC2, and MUC5AC in tumor samples from hepatocellular carcinoma patients with cardiac metastasis was assessed using immunochemistry. Results were compared to findings for a group with a better prognosis - those with primary hepatocellular carcinoma without recurrence. Results: Pathologic examinations indicated that patients with hepatocellular carcinoma with cardiac metastasis had more vascular invasion ($P=0.004$) and less section free zone involvement ($P<0.001$) than those with primary hepatocellular carcinoma. MUC1 expression was significantly higher in hepatocellular carcinoma with cardiac metastasis ($P<0.005$). Conclusions: Expression of mucins, especially MUC1, confirms the malignant nature of hepatocellular carcinoma with cardiac metastasis. It is recommended that these patients receive aggressive therapy.

2193/B662

Mirna Profile of Tumor Progression in Ovarian Carcinoma.

O. Vaksman[1], B. Davidson[2], R. Reich[1]; [1]Pharmacology, Hebrew University, Jerusalem, Israel, [2]Pathology, Norwegian Radium Hospital, Oslo, Norway

MicroRNAs (miRNAs) are small non-coding RNAs. They regulate gene expression by binding to the 3'UTR of a target mRNA, and thus decrease protein levels by degradation of the mRNA, or by inhibiting protein translation. In the recent years, miRNAs were found to play an important role in cancer, and suggested to have better prognostic value than gene arrays. Ovarian cancer is the leading cause of death from gynecological cancer in Western countries. The disease is asymptomatic in the early stages, and is usually diagnosed at an advance stage. Primary solid tumor, solid metastases, and effusions to the peritoneal and pleural cavities characterize the tumor as it progresses. The aim of the study was to characterize the difference in miRNA expression pattern between solid primary and effusion-derived tumors, using freshly frozen samples. Using microRNA-array platforms, we identified three sets of miRNAs: one set highly expressed in both primary solid tumors and in effusions, one set relatively upregulated in effusions, and one set relatively downregulated in effusions. The most significant miRNAs were validated by real-time PCR. Our results show concordance between the training and the independent test cohorts for the downregulated miR-125b and for the upregulated miR-222, miR-210 and miR-200c in effusions. Using target prediction programs to identify potential targets for the above miRNAs, we found PAK1 to be a putative target of miR-222. Indeed, we found PAK1 to be down regulated in effusions compared to solid tumors, initiating a signaling cascade that culminate in E-cadherin upregulation in these tumors. Similarly, miR-200c, targets the transcription factor TCF8 and by doing so, upregulates further E-cadherin levels. In summary, miRNA expression profiles can be used for prognosis in ovarian carcinoma.

2194/B663

Critical Genetic Changes in HPV-Related and Unrelated Oropharyngeal Carcinoma and Their Prognostic Implications.

C. Huebbers[1], J. Klussmann[2]; [1]Jean-Uhrmacher-Institute for Oto-Rhino-Laryngological Research, University of Cologne, Cologne, Germany, [2]Department of Oto-Rhino-Laryngology, Head and Neck Surgery, University of Cologne, Cologne, Germany

Patients with human papillomavirus (HPV)-containing oropharyngeal squamous cell carcinomas (OSCC) have a better prognosis than patients with HPV-negative OSCC. This may be attributed to different genetic pathways promoting cancer. We used comparative genomic hybridization (CGH) to identify critical genetic changes in 60 OSCC, 28 of which were associated with HPV-16 as determined by HPV-specific PCR and FISH analysis and positive p16INK4A immunostaining. Results were correlated with HPV status and clinical patient data. Two third of OSCC harbored gain at 3q26.3-qter irrespective of HPV status. In HPV-negative tumors this alteration was associated with

advanced tumor stage (P=0.013). In comparison with HPV-related OSCC the HPV-negative tumors harbored: a) a higher number of chromosomal alterations and amplifications (P=0.03 and 0.039, respectively), b) significantly more losses at 3p, 5q, 9p, 15q and 18q and gains/amplifications at 11q13 (P=0.002, 0.03, <0.001, 0.02, 0.004 and 0.001, respectively), and c) less often 16q losses and Xp gains (P=0.02 and 0.03). Survival analysis revealed a significantly better disease-free survival for HPV-related OSCC (P=0.02), whereas chromosome amplification was an unfavorable prognostic indicator for disease-free and overall survival (P=0.01 and 0.05, respectively). Interestingly, 16q loss, predominantly identified in HPV-related OSCC, was a strong indicator of favorable outcome (overall survival: P=0.008, disease-free survival P=0.01) and none of these patients had a tumor recurrence. Genetic signatures of HPV-related and -unrelated OSCC are different and most likely underlie differences in tumor development and progression, In addition, distinct chromosomal alterations have prognostic significance.

2195/B664

A Novel Method to Capture Methylated Human DNA from Urine: Implications for Prostate Cancer Screening.

A. Haj-Ahmad[2], C. Dobbin[1], Y. Haj-Ahmad[2,1]; [1]Norgen Biotek Corp., Thorold, ON, Canada, [2]Brock University, St. Catharines, ON, Canada

Prostate cancer is the most commonly detected male cancer and the second leading cause of death in the United States. Diagnosis and management are confounded by the lack of symptoms and the lack of cancer-specific diagnostic techniques to be used during early stages of the disease. Novel approaches for the early detection and management of prostate cancer are therefore urgently needed. Hypermethylation of regulatory sequences at the glutathione S-transferase pi (GSTP1) gene locus is found in the majority (>90%) of primary prostate carcinomas, but not in normal prostatic tissue or other normal tissues. In addition, urine from prostate cancer patients is found to contain shed neoplastic cells and DNA amenable to molecular analyses. The development of a non-invasive test for the molecular diagnosis of prostate cancer from urine therefore appears to be feasible and is the goal of this study. In particular, the isolation and enrichment of hypermethylated DNA directly from urine is used as a prostate cancer specific biomarker for downstream molecular diagnostic assays. In this study we evaluated the effectiveness of several urine-DNA isolation technologies on the enhancement of the sensitivity and specificity of non-invasive, urine-based point-of-care diagnosis of prostate cancer. Specific aims of the project were: (1) the identification and elimination of inhibitors associated with urine DNA, thereby consistently purifying biologically active DNA that can be readily used in diagnostics; (2) the improvement of current methods in order to allow the separation and enrichment of methylated DNA from urine for use in cancer diagnostics; (3) the determination if methylated DNA isolated from urine can be of diagnostic value in the early detection of prostate cancer. We hypothesized that the existing molecular tests for methylated DNA, including methylation specific PCR, can be made more sensitive and specific by improving upon the sample preparation of methylated DNA from urine.

Metabolic Diseases II (2196 – 2210)

2196/B667

Dissection of the Major Cellular Pathways for PCSK9-Induced LDLR Degradation.

S. Poirier[1], G. Mayer[1], V. Poupon[2], P. McPherson[2], N. G. Seidah[1]; [1]Biochemical Neuroendocrinology, Clinical Research Institute of Montreal, IRCM, Montreal, QC, Canada, [2]Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, QC, Canada

Introduction: A high level of circulating LDL-cholesterol (LDL-C) is a major risk factor for coronary heart disease (CHD). We recently identified the convertase PCSK9 as the third locus for autosomal dominant familial hypercholesterolemia (ADH). More recently it was shown that loss-of-function mutations in PCSK9 resulted in an ~88% reduction in CHD which correlates with a strong decrease in circulating LDL-C. Binding of hepatic PCSK9 to intra- or extracellular low-density lipoprotein receptor (PCSK9-LDLR) routes the receptor from the recycling pathway towards its degradation in late endosomes/lysosomes (E/L), resulting in an increased circulating LDL-cholesterol in the plasma. Results and Conclusions: Little is known about the mechanisms and the relative contributions of the intracellular versus extracellular pathways for PCSK9-induced LDLR degradation. Using HepG2 cells, we showed either by immunocytochemistry or FACS analyses that the gain-of-function mutant PCSK9-D374Y, but not the wild-type PCSK9, lowers the level of cell surface LDLR on neighboring cells. In order to define the contribution between intra- versus extracellular PCSK9 on LDLR degradation, we used specific siRNAs to silence proteins that affect general cellular trafficking and LDLR endocytosis specifically. Thus, our data reveal that extracellular PCSK9 provides a minor contribution to LDLR intracellular degradation. Compared to a PCSK9-negative cell line (HEK293), we show that disruption of intracellular trafficking in HepG2 cells strongly increases LDLR protein levels without affecting its endocytic activities. This study demonstrates for the first time that intracellular PCSK9 in HepG2 cells (but not in HEK293 cells) plays an important role in enhancing LDLR degradation, providing new information on the subcellular site(s) of action of PCSK9, a key emerging target in the treatment of hypercholesterolemia and CHD.

2197/B668

Nogo-B Receptor Interacts with Niemann-Pick Type C2 Protein and Regulates Intracellular Cholesterol Trafficking.

K. D. Harrison, R. Miao, C. Fernandez-Hernando, Y. Suarez, A. Davalos, W. C. Sessa; Pharmacology, Yale University, New Haven, CT

The 293-residue Nogo-B Receptor (NgBR) is a novel receptor for the N-terminus of Nogo-B (AmNgB). Other than its role in AmNgB binding, much remains unknown concerning the precise function of NgBR. Sequence analysis of NgBR reveals a single C-terminal domain with homology to cis-isoprenyltransferase, an enzyme responsible for successive condensation of isopentenyl disphosphate to form long-chain polyprenols. We sought an understanding of a basic cellular role for NgBR by performing a yeast-2-hybrid assay for interacting proteins using the cytosolic 158-residue cis-isoprenyltransferase domain as bait. We identified Niemann-Pick Type C2 protein (NPC2) as an NgBR-interacting protein. NPC2 is a cholesterol-binding protein that is necessary for efficient intracellular trafficking of LDL-derived cholesterol. Mutations in the NPC2 gene lead to a lysosomal storage disorder classified as Niemann-Pick Type C2 disease. We show here that NPC2 colocalizes and coimmunoprecipitates with NgBR in mammalian cells. NPC2 protein levels are increased in the presence of

NgBR and NgBR enhances NPC2 protein stability. NgBR localizes primarily to the endoplasmic reticulum (ER), and colocalizes with a portion of NPC2 in the ER. RNAi-mediated disruption of NgBR expression leads to a decrease in NPC2 levels, increased intracellular cholesterol accumulation and a loss of sterol sensing, all hallmarks of NPC2 mutation. Moreover, mouse embryonic fibroblasts heterozygous for NgBR expression display increased free cholesterol levels by filipin staining. NgBR-dependent defects in intracellular cholesterol accumulation are rescued by addition of exogenous NPC2, treatment with oxysterols, and by ectopic NgBR expression. Analysis of NgBR expression in NPC disease-derived fibroblasts reveals a decrease in NgBR protein levels, providing evidence of a possible cooperative relationship between NPC2 and NgBR. These data identify NgBR as a new NPC2-interacting protein and provide evidence of a novel role for NgBR in intracellular cholesterol trafficking.

2198/B669

Biological Candidate Markers of Fabry Disease: a Genomic Approach Using a Mouse Model.

J. Choi, E. Park, H. Park, S. Jung; Ewha Womans University, Seoul, South Korea

Fabry disease is a lysosomal storage disease caused by a deficiency of a-galactosidase A, which results in aberrant glycosphingolipid metabolism and accumulation of globotriaosylceramide(Gb3). As correlations between the level of Gb3 and clinical manifestations of Fabry disease have not been developed, we investigated potential diagnostic biomarkers. Hepatic and renal gene expression of male a-galactosidase A deficient mice (Fabry mice) was compared with that of wild-type mice. Microarray analyses were performed using samples taken before and after intravenous infusion of a-galactosidase A. The identified genes were validated using quantitative real-time PCR and Western blotting. Expression of hepatic Saa1 (serum amyloid A1), S100a8 (S100 calcium-binding protein A8), S100a9 (S100 calcium-binding protein A9) and Lcn2 (lipocalin 2) and renal Npy (neuropeptide Y) was significantly up-regulated in Fabry mice compared with wild-type mice and was normalized by enzyme replacement therapy. Plasma concentrations of Saa1, Lcn2 and Npy were greater in Fabry mice than in wild-type mice. The genes identified may play important roles in the pathogenesis of Fabry disease and are promising targets for the development of biomarkers for monitoring disease progression and therapeutic efficacy in patients with Fabry disease.

2199/B670

Chronic Alcohol Consumption Leads to Global Protein Hyperacetylation and Impaired Hepatic Function.

B. D. Shepard[1], D. J. Fernandez[1], D. J. Tuma[2], P. L. Tuma[1]; [1]Biology, The Catholic University of America, Washington, DC, [2]Department Of Internal Medicine, University of Nebraska Medical Center, Omaha, NE

While alcoholic liver disease is clinically well-described, the molecular basis for alcohol-induced hepatotoxicity is not well understood. We have been using the polarized, hepatic WIF-B cells for studies on alcohol toxicity. We have shown that WIF-B cells display alcohol-induced defects in cell processes as described in situ. These include (so far) decreased tubulin polymerization and defects in endocytosis and secretion. We also discovered that microtubules in alcohol-treated WIF-B cells (and in alcohol-treated rat livers) were hyperacetylated and more stable, and was caused by impaired HDAC6 (a known microtubule deacetylase) microtubule binding. We proposed that the ethanol-induced alterations in microtubule dynamics may explain the impaired protein trafficking. To test this, we used the HDAC6 inhibitor, trichostatin A (TSA), that leads to increased microtubule acetylation and stability in the absence of ethanol. We found that both ethanol and TSA impaired secretion and selectively impaired clathrin-mediated endocytosis confirming our hypothesis. Most recently, we have shown that the microtubule-dependent nuclear translocation of STATs 3 and 5 is impaired in ethanol-treated WIF-B cells and in livers of ethanol-fed rats. Because ethanol induces hyperacetylation of other proteins including histone H3 and p53, we initiated a search to identify novel hyperacetylated proteins using a proteomics approach. We prepared nuclear, cytosolic, and total membrane fractions from control and ethanol-fed rat livers and blotted them with anti-acetylated lysine antibodies. In all fractions, numerous ethanol-induced hyperacetylated proteins were detected. Cytosolic and membrane fractions were further analyzed by 2D gels, immunoblotting and mass spectrometry of 50 selected spots. Many of the proteins were metabolic enzymes or proteins involved in regulating oxidative stress. The latter population is of particular interest because oxidative stress is induced by alcohol metabolism. Also, β-actin was identified whose γ isoform is already known to be acetylated. We are currently confirming the ethanol-induced hyperacetylation of selected proteins and are examining the affects of acetylation on hepatic function.

2200/B671

Involvement of AMP-Activated Protein Kinase/ACC Pathway on the Fatty Liver Induced by Orotic Acid.

E. Jung, Y. Lee, S. Yoon, B. Lee; College of Pharmacy, Seoul National University, Seoul, South Korea

Orotic acid (OA) has been known to cause fatty liver with poorly defined cellular mechanism of action. AMP- activated protein kinase (AMPK) is a critical regulator of cellular and body energy homeostasis sensing the status of the AMP:ATP ratio. The net effect of phosphorylation of AMPK by AMPK kinase in the liver is stimulation of fatty acid oxidation and inhibition of lipogenesis. The present study was aimed to investigate whether the OA-induced fatty liver is mediated by AMPK phosporylation. OA induced intracellular lipid accumulation in SK-Hep1 human hepatoma cells in a dose- and time-dependent manner. Exposure of SK-Hep1 cells to OA significantly reduced the Thr-172 phosphorylation of AMPK, and also repressed the phosphorylation of acetyl-CoA carboxylase (Acc), a well-known substrate of AMPK. Inhibition of AMPK phosphorylation and lipid accumulation by OA was reversed by 5-amino-4-imidazolecarboxamide riboside (AICAR). Expression of LKB1 was decreased by OA without significant change in the intracellular AMP:ATP ratio. We also investigated the effects of OA on the transcriptional repression of PPAR-α and the expression of the target genes using PPRE-reporter gene assay and quantitative real-time PCR in H4IIEC3 cells. Target genes for PPAR-α include acyl-CoA oxidase (Acox), acyl-CoA dehydrogenase, short/branched chain (Acadsb), L-3-hydroxyacyl-CoA dehydrogenase, short chain (Hadhsc) and acyl-CoA dehydrogenase, very long chain (Acadvl). Treatment of H4IIEC3 cells with OA repressed the transcriptional activity of PPAR-α by 20%. Accordingly, OA inhibited the expression of Acox, Acadsb, Hadhsc, and Acadvl by 32%, 22%, 40% and 24% respectively. These results indicate that the inhibition of AMPK-dependent anti-lipogenic pathway and the inhibition of PPAR- α-dependent fatty acid oxidation mechanism contribute to the steatogenic effects of OA.

2201/B672

Second-Hand Smoke Stimulates Lipid Accumulation in the Liver by Inhibiting 5'-AMP-Activated Protein Kinase Activity.

H. Yuan[1], J. Shyy[2], M. Martins-Green[1]; [1]Cell Biology and Neurosciences, University of California, Riverside, Riverside, CA, [2]Division of Biomedical Sciences, University of California, Riverside, Riverside, CA

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of a metabolic syndrome. The underlying mechanisms of fatty liver, the first stage of NAFLD that is revealed by the accumulation of lipid in hepatocytes, remain unclear. Cigarette smoke is known to change circulating lipid profiles of smokers, thus may also contributed to the accumulation of lipids in the liver and to the initiation of NAFLD. To test this possibility, mice were exposed to sidestream whole (SSW) smoke, a major component of "second-hand" smoke, and cultured hepatocytes were exposed to SSW solutions. A variety of cells and molecular approaches were used to show the effects of SSW on the changes of the activities of 5'-AMP-activated protein kinase (AMPK) and sterol response element binding protein-1 (SREBP-1), which are two critical molecules involved in lipid metabolism. The results show that compared to fresh air or media, SSW causes lipid accumulation in hepatocytes both in vivo and in vitro by modulating the activity of these two molecules. SSW causes dephosphorylation/inactivation of AMPK which results in increased activation of SREBP-1and subsequently leads to accumulation of triglyceride in hepatocytes. The findings that cigarette smoke stimulates lipid accumulation in hepatocytes in vivo and that AMPK inactivation results in SREBP-1 activation are novel and important because they point to another risk factor of smoking, i.e., that of contributing to fatty liver disease and to two new molecular targets of cigarette smoke, revealing a new mechanism for cigarette smoke-induced lipid accumulation in the liver. The elucidation of this mechanism may help us understand processes involved in atherogenesis and in initiation of NAFLD, and suggest possible ways of treating both of these metabolic diseases. It will also be helpful in developing new treatment for metabolic diseases in general, such as diabetes and obesity.

2202/B673

High Content Interrogation of Lipid Droplet Accumulation Control Machinery in Human Adipocytes.

S. M. Hartig[1], D. S. Loose[2], A. T. Szafran[1], I. P. Uray[1], B. Buehrer[3], R. Giersch[3], M. Mancini[1]; [1]Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX, [2]Integrative Biology and Pharmacology, University of Texas Health Science Center, Houston, TX, [3]Zen Bio, Inc., Research Triangle Park, NC

The dominant cellular basis for obesity is increased accumulation of triglycerides in lipid droplets leading to an escalation in adipocyte size. Requisite for the adipocyte differentiation program are several nuclear receptors: peroxisome proliferator-activated receptor (PPAR), androgen receptor (AR), estrogen receptor (ER), their coregulators (e.g. SRC-1, SRC-2, SRC-3). However, little is known about how these genes coordinately function in all phases of human adipocyte differentation, especially in light of variable findings on ER and AR-mediated adipogenesis. We have examined gene expression profiles of all 49 nuclear receptors and available coregulators (~300). The gene chips revealed that in addition to PPAR upregulation, AR levels were highly increased during human adipocytes differentiation while ER was constant. Additionally, SRC-3 transcripts increased while SRC-1 and SRC-2 levels remained unchanged. To further quantitatively profile the complementary role of nuclear receptors and coregulators during adipogenesis in a cellular context, our lab has developed a new high content microscopy-based technology coupled with advanced image data mining protocols (Pipeline Pilot, Accelrys, Inc.). This systems biology approach, coupled with RNAi, establishes a cytological fingerprint in human adipocytes for these 6 nuclear receptors and coregulators. A multiplex variable set is applied including: lipid droplet formation, lipid binding protein trafficking, receptor and coregulator activity, subnuclear morphology, and cell cycle analyses. Further, preliminary data has indicated that siRNA knockdown of SRC-1, SRC-2, and SRC-3 affects lipid accumulation and cell cycle progression in the earliest stages of human adipogenesis (72 h). In conclusion, this ultra-high content image analysis approach can be a powerful tool to evaluate crosstalk amongst nuclear receptors and coregulators.

2203/B674

Obese Diet Enhances Free Fatty Acid in Bone Marrow.

H. Choi, Y. Kim; Department of Biological Sciences and the Immunomodulation Research Center, University of Ulsan, Ulsan, South Korea

Obesity is considered to be state of chronic inflammation that leads to metabolic derangements. Increased adipose tissue mass is associated with changes in the endocrine and metabolic functions of adipose tissue. Free fatty acids (FFA) are generated by adipocytes, in which stored triglycerides are cleaved by lipolysis. FFA play an essential role as signaling molecules of the chronic inflammatory disease associated with obesity and hyperlipidemia, although the molecular mechanism as to how they modulate the development of chronic diseases has not been clearly understood. Epidemiological studies link hyperlipidemia with osteoporosis. Patients with lower bone density and osteoporosis have higher lipid levels, and osteoporosis has been connected to increased marrow fat content, suggesting a systemic interaction between fat and bone metabolism, and free fatty acid in the bone marrow could cause bone loss. Our results also demonstrate that obesity is highly associated with lower bone density. Obesity results in higher level of FFA in bone marrow. Elevated FFA could be responsible for increased osteoclastogenesis in bone marrow cells from obese mice. This work was supported by This work was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-070-C00249) and KOSEF grant funded by the Korea government (MOST) (R01200700021 08202007).

2204/B675

Tumor Necrosis Factor (TNF) Antagonists Improve High Cholesterol, Tryglyceride, HbAlc and MMP-3 Levels. A Different Effect between TNF Antagonists from DMARDs.

T. Imaoka[1,2], K. Tsubone[1], M. Okamoto[1], K. Kanoh[2], M. Imaoka[2], T. Imaoka[2], N. Takahashi[2]; [1]Biochemistry and Immunology and Bioscience, Misuzugaoka Higashi Clinic, Hiroshima, Japan, [2]Allergy and Rheumatology, Yoshimien Coast Park Clinic, Hiroshima, Japan

Platelet pleckstrin (47K) phosphorylation by protein kinase C is closely associated with degranulation (T.Imaoka and J.Haslam, J.Biol. Chem, 258, 11404). Pleckstrin contains an unique PH domain which associates with R subunit of GTP binding protin, then stimulate phospholipase C activity. We first purified pleckstrin, protein kinase C, protein phosphatase 2A to homogeneity from human platelets. Anti-Rhueumatic drug, DMARDs (CCA etc) inhibited protein phosphorylation and stimuleted dephosphorylation reaction using Histone1, 2B, and

unphosphorylated pleckstrin, then inhibited cell functions. On the other hand, TNF antagonists improved high cholesterol, triglyceride, HbAlc and MMP-3 levels in peripheral blood after TNF antagonists with Methotrexate (MTX) combination therapy in a total of 240 Rheumatoid Arthritis (RA) patients, possibly as a result of the stimulation of cell functions. This observation suggests that 1) TNF would be an important regulatory factor in the systemic metabolism, and that 2) TNF antagonists have a different effect from the effects of DMARDs, via protein phosphorylation, or others.

2205/B676

A Possible Role of Transthyretin Related to the Pathogenesis of Phenylketonuria.

J. Park, E. Park, E. Choi, H. Park, S. Jung; Department of Biochemistry, School of Medicine, Ewha Womans University, Seoul, South Korea

Phenylketonuria (PKU) is an autosomal recessive disorder caused by a deficiency of phenylalanine hydroxylase (PAH), which catalyzes the conversion of phenylalanine to tyrosine. The resultant hyperphenylalaninemia causes mental retardation, seizure, and abnormalities in behavior and movement. Gene expression profiling of brain tissue from a mouse model of PKU revealed overexpression of transthyretin (Ttr), sclerostin domain containing 1 (Sostdc1), alpha-Klotho (Kl), prolactin receptor (Prlr), and early growth response 2 (Egr2). The upregulated state of these genes in brain tissue was confirmed using real-time PCR and Western blotting. In contrast to its overexpression in the brain, TTR expression was low in the sera of PKU mice offered unrestricted access to a diet containing phenylalanine. Expression of TTR decreased in a time-dependent manner in 0.9 mM phenylalanine-treated HepG2 cells and in 1.2 mM phenylpyruvate-treated HepG2 cells. Treatment with 0.9 mM phenylalanine or with 1.2 mM phenylpyruvate decreased transcriptional activity of transthyretin promoter site in HepG2 cells. These findings indicate that Ttr and other genes play important roles in the pathogenesis of PKU and that phenylalanine and its metabolites, phenylpyruvate and phenyllactate might have a direct effect on the level of TTR in serum.

2206/B677

Subcellular Localization of UDP-Glcnac 2-Epimerase/Mannac Kinase, the Key Enzyme in Sialic Acid Synthesis.

K. Patzel[1], M. Anahtar[1], C. Ciccone[1], H. Dorward[1], I. Manoli[1], D. Adams[1], T. D. Butters[2], W. A. Gahl[1], M. Huizing[1]; [1]Medical Genetics Branch, NHGRI, Bethesda, MD, [2]Biochemistry, University of Oxford, Oxford, United Kingdom

UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE/MNK) is a rate limiting, bifunctional enzyme in sialic acid (SA) synthesis, which is feedback inhibited by CMP-SA in its allosteric site. Mutations in GNE/MNK cause two human disorders, Hereditary Inclusion Body Myopathy (HIBM) and sialuria. Sialuria, clinically characterized by variable symptoms, including hepatomegaly and mental retardation, is caused by dominant mutations in the allosteric site of GNE/MNK, leading to a loss of feedback-inhibition and increased excretion of SA. HIBM is characterized by adult onset progressive muscle wasting and results from recessive, mostly missense, mutations in GNE/MNK outside the allosteric site, leading to decreased GNE/MNK enzyme activities, and decreased SA production. Recent immunofluorescence studies showed that GNE/MNK not only resides in the cytoplasm and Golgi, as expected, but also in the nucleus. This nuclear localization is remarkable, since GNE/MNK performs its enzymatic role within the cytoplasm. However, GNE/MNK contains a putative nuclear export signal and the nucleus contains CMP-SA synthase, which converts all cellular SA to CMP-SA. We propose GNE/MNK is 'stored' in the nucleus, in its inactive, CMP-SA bound, feedback-inhibited form. If the cell needs SA, there is less SA to enter the nucleus and form CMP-SA, so there is more 'free' GNE/MNK to translocate to the cytoplasm (through its nuclear export signal) and produce more SA. To study this proposed mechanism, we obtained preliminary data using normal, HIBM and sialuria patient's fibroblasts. Pilot immunofluorescence studies revealed a higher nuclear GNE/MNK content in sialuria cells and a lower content in HIBM cells compared to normal. Subcellular fractionation followed by Western blotting revealed similar results; higher nuclear amounts of GNE/MNK in sialuria and lower amounts in HIBM. In addition, Western blots of nuclear fractions of both sialuria and HIBM cells showed unexplained GNE/MNK bands compared to normal. These bands, as well as the function of the nuclear export signal, are being analyzed and may provide intriguing insights into the regulation of cellular SA synthesis.

2207/B678

Differences in Transcription and Splicing between Human INPP5B and Mouse Inpp5b.

S. P. Bothwell, L. W. Farber, R. L. Nussbaum; Institute for Human Genetics, University of California at San Francisco, San Francisco, CA

Lowe syndrome is an X linked disorder characterized by congenital cataracts, renal Fanconi syndrome, and variable degrees of mental retardation. It is caused by loss-of-function mutations in the *OCRL1* gene, a PIP2 5-phosphatase. However, *Ocrl1* knockout mice do not show a phenotype. *Inpp5b* is an autosomal gene encoding another PIP2 5-phosphatase and is a very close paralog of *Ocrl1*. *Inpp5b* knockout mice also do not show a Lowe syndrome phenotype, but mice with knockouts of both *Ocrl1* and *Inpp5b* are synthetic lethals and die prior to implantation, indicating functional overlap. We hypothesize *Inpp5b* may compensate completely for the loss of ocrl1 in mice while *INPP5B* can only partially compensate in humans, leading to the Lowe syndrome. We therefore decided to investigate how human *INPP5B* and mouse *Inpp5b* differ. First, *INPP5B* is expressed at lower levels. Second, *INPP5B* utilizes a GC splice site within exon 7, resulting in a transcript that is 80 amino acids shorter than the transcript in mice. We also discovered that both the human and mouse genes generate a second transcript that begins in exon 7 from an internal promoter and showed that sequences from either human or murine exon 7 had significant promoter activity by luciferase assay when cloned into a promoterless vector. The presence of a second transcript corresponding to an internal promoter was further confirmed by Northern blot. The collective results indicate there is an internal promoter within both murine *Inpp5b* and human *INPP5B*. We hypothesize that the difference between the paralogs in their ability to compensate for loss of Ocrl1 may be multifactorial and include quantitative differences in promoter strength of the two transcripts as well as a qualitative difference in splicing. For this reason, we have chosen to develop a mouse model of Lowe syndrome by creating a mouse that is deficient in murine *Ocrl1* and humanized at the *Inpp5b* locus.

2208/B679

Mutagenic Studies of the Intermonomeric Interactions in Human Arginase, an Enzyme Involved In Regulation of the Cellular Levels

of Arginine.

N. Carvajal, M. Lobos, E. Uribe, D. García; Molecular Biology, University of Concepcion, Concepcion, Chile

Arginase, a trimeric metalloenzyme that catalyses the hydrolysis of arginine to ornithine and urea, participates in ureagenesis and regulation of cellular levels of arginine, a precursor for creatine, proline, polyamines and the cell-signaling molecule nitric oxide. This makes arginase a potential clinical marker and target for pharmacological intervention of NO-dependent smooth muscle disorders (vascular disease, asthma, erectile disfunction). Also biologically and clinically relevant is liver arginase deficiency, an autosomal recessive disorder that may compromise active site residues and even the trimer stability. A deep knowledge of the cellular and molecular aspects of arginase is therefore needed. To contribute, we are examining the intersubunit interactions by using a mutagenic approach. After observing the loss of cooperative effects with respect to arginine by truncation of the last 14 residues of a C-terminal S-shaped motif (residues 304-322, which account for about 54 % of the intersubunit interactions in human arginase) and the monomeric state of the R308A mutant, we now mutated Asp204 and Glu256, which respectively establish intermonomer salt linkages with Arg308 and Arg255. Mutants D204A and E256Q were monomers (30 kDa), whereas E256A was trimeric (99 kDa). Tryptophan fluorescence was not altered. Kinetics at pH 7.5 was cooperative for wild-type arginase (Hill coefficient 1.7) and hyperbolic for all the mutants. In contrast with conversion of monomeric R308A to kinetically cooperative trimers by 2 mM guanidine at pH 7.5, all other species were not altered. At the optimum pH (9.5), kinetics for wild-type and mutants was hyperbolic, with equal K_m (5-7 mM), k_{cat} (180-190 seg^{-1}) and K_i values for ornithine (1-3 mM) and guanidine (50-60 mM). Results agree with guanidine mimicking the guanidine group of Arg308 at the intermonomer interface and discard elimination of a negative charge as explanation for monomerization of E256Q. Results also shows that not only the entire S-shaped motif, but also Glu256 is essential for the cooperative response of the enzyme to arginine. Grant FONDECYT 1070467

2209/B680

Involvement of Autocrine EGF-Receptor Ligands in the Palmitate-Induced COX-2 Expression in C2C12 Myotubes.

A. Kadotani[1,2], H. Katagiri[2], M. Kanzaki[1]; [1]Center for Research Strategy and Support (CRESS), Tohoku University, Sendai, Japan, [2]Division of Advanced Therapeutics for Metabolic Diseases, Center for Translational and Advanced Animal Research, Tohoku University, Sendai, Japan

Skeletal muscle has been reported to produce soluble mediators, called myokines (eg, IL-6 and TNF-alpha), in response to free fatty acid and/or muscle contraction. However, little information is available about whether other secreted proteins, such as growth factors, are produced by skeletal muscle. In this study, we evaluated the effects of palmitate on production of epidermal growth factor (EGF) family ligands, such as amphiregulin (AREG) and epiregulin (EREG), and elucidated the role of these EGF-R ligands in palmitate-induced COX-2 expression in C2C12 myotubes. Measurement of AREG and EREG in culture supernatants by ELISA revealed that palmitate treatment (16 h) induces expression and production of AREG and EREG, accompanied by enhanced expression of cyclooxygenase (COX)-2 in myotubes. Release of AREG and EREG in the culture supernatants was completely inhibited by TAPI-1 (TNF-alpha converting enzyme [TACE] inhibitor), suggesting these EGF-R ligands to be converted to soluble mature forms by TACE in myotubes. Pharmacological inhibition of EGF-R activation led to partial reduction of COX-2 expression, while inhibition of COX-2 failed to affect production of AREG and EREG, suggesting that these autocrine EGFR ligands at least partially mediate the palmitate-induced COX-2 expression. Western blot analysis showed the possibility that cAMP responsive element binding protein may be involved in the palmitate-induced expression of AREG and EREG, and that p38 and NF-kappa B is required for the COX-2 expression. We also found that, unlike palmitate, a monounsaturated fatty acid oleate had no ability to induce expression of AREG and EREG as well as of COX-2, but instead oleate completely abolished the palmitate-induced expression of AREG, EREG and COX-2. Together, these data indicated that AREG and/or EREG may regulate COX-2 expression via activation of EGF-R (erbB) family in an autocrine fashion. Thus, our data shed light on the potential link between the palmitate-induced inflammatory responses and the autocrine EGFR ligands released by TACE-mediated shedding in skeletal muscle cells.

2210/B681

Lauric Acid Enhance Osteoclast Survival by MIP-1Alpha.

H. Choi, O. Sul, H. Kim; Department of Biological Sciences and the Immunomodulation Research Center, University of Ulsan, Ulsan, South Korea

Hyperlipidemia and bone marrow adipogenisty are reported to reduce bone density in vivo, suggesting the relationship between fat and bone metabolisms. Elevated osteoclastogenesis occurs in bone marrow-derived macrophage (BMM) cells by lauric acid. Lauric aicd enhances osteoclast (OC) survival through activation of nuclear factor (NF)-κB, and increases expression level of MIP-1-α. OC formation and OC survival were evaluated in BMM, and mature OCs, respectively. Apoptosis of mature OCs was evaluated by observing the degradation of DNA. Activation of NF-κB was measured by electrophoretic mobility shift assay. Lauric acid stimulated receptor activator of nuclear factor-κB ligand (RANKL)-induced OC formation by acting on OC precursor cells, and prevented OCs from apoptosis, generating MIP-1α. This work was supported by This work was supported by the Korea Research Foundation Grant funded by the Korean Government (KRF-2008-070-C00249) and KOSEF grant funded by the Korea government (MOST) (R01200700021 08202007).

Neuronal Diseases II (2211 – 2229)

2211/B682

The Drosophila Neurodegenerative Mutant Blue Cheese Links Lipid Metabolism with Lysosomal Function.

S. Hebbar[1], A. Lim[2], R. S. Kraut[1]; [1]IBN, A-STAR, Biopolis, Singapore, Singapore, [2]Dept of MCD Biology, University of California, Santa Cruz, Santa Cruz, CA

Many studies suggest a link between sphingolipid metabolism and neurodegeneration in humans. The objective of our work is to understand the relationship between sphingolipid activity and neurodegeneration, using Drosophila genetics. We report on the characterization of blue cheese (bchs), a lysosomal protein with a conserved BEACH domain. This motif is required for sphingomyelinase (SMase) activation by the

protein FAN (Factor Activating Neutral-SMase) in response to stress signals. Overexpression or loss of function of Bchs results in neurodegeneration in the larval motor nervous system and other neuronal tissues (Lim and Kraut, submitted; Finley et al, 2003). Using wide-field fluorescence imaging of motorneurons in intact animals, we documented a breakdown in anterograde lysosomal axon transport in bchs-. Additionally, we find alterations in total brain sterol levels and in the relative levels of free vs. esterified cholesterol in bchs animals, using a fluorimetric enzymatic assay (Amplex Red). Bchs' possible role in sphingomyelin cleavage raises the possibility that cholesterol metabolism is regulated by modulations in the levels of a SM-like lipid in the fly, PE-ceramide. Consistent with this idea, genetic evidence links bchs-induced neurodegeneration to altered sphingolipid metabolism, with ceramide levels being the critical factor in regulating the extent of degeneration. Overexpression or loss of function in ceramidase or SMase modulate the degenerative phenotype. Currently, very little is known at the cell biological level about the role of sphingolipids and cholesterol processing and trafficking in neurodegeneration in the fly (but see Acharya, '03; Tschape '02; Phillips, '08). However, experiments done in our lab on Drosophila neuronal cells in culture suggest that the regulation of sphingolipid trafficking by cholesterol may be similar in Drosophila and mammals. In summary, our characterization of motorneuron degeneration in bchs shows that an interaction between sphingolipid and cholesterol metabolism is likely to exist in flies, and may be involved in the progression of neurodegeneration, similar to what is seen in human diseases such as Alzheimer's, ALS, and the lysosomal storage diseases.

2212/B683

Purkinje Cell Neurodegeneration in the Pkcγ H101Y Transgenic Mouse.

A. K. Snider[1], Y. Zhang[2], D. Takemoto[1], D. Lin[2]; [1]Biochemistry, Kansas State University, Manhattan, KS, [2]Human Nutrition, Kansas State University, Manhattan, KS

Objectives: Spinocerebellar ataxia type 14 (SCA14) is an autosomal, dominant neurodegenerative disorder caused by mutations in protein kinase C gamma (PKCγ), with onset ages of three to thirty years in humans. PKCγ is a neuronal specific PKC. Activated PKCγ phosphorylates and inhibits gap junctions such as Cx57, a process which is controlled by the cell oxidative state. We have previously demonstrated that PKCγ SCA14 mutations lead to endoplasmic reticulum (ER) stress and apoptosis through "out of control" gap junctions in HT22 cells in culture. The objective of this study is to determine effects of H101Y mutation in transgenic mice in vivo on Purkinje cells. Methods: In this study, over-expressed HA-tagged PKCγ H101Y SCA14 mutant was generated in a C57BL/6J background to produce a SCA14 transgenic mouse model. Brain sections were analyzed using immunocytochemistry, light microscopy, enzyme assays and western blots. Results: Immunohistochemistry results demonstrated that similar Purkinje cell localization of HA-tagged PKCγ H101Y mutant proteins is found in the PKCγ H101Y transgenic mouse compared to the control mouse. Light microscopy of sections from saggital cerebella of the control and PKCγ H101Y transgenic mice showed altered morphology and loss of Purkinje cells at postnatal four weeks in the mutant mice. Biochemical studies further demonstrated that PKCγ H101Y SCA14 mutation causes lack of total cellular PKCγ enzyme activity, loss of the gap junction protein, Cx57 phosphorylation on serines, and activation of caspase-3 and caspase-12 in the PKCγ H101Y SCA14 transgenic mouse. Failure of stereotypical clasping responses in the hind limbs of the transgenic mice was also observed. Conclusion: This is the first transgenic mouse in existence which models a human SCA14 mutation and results clearly demonstrate a need for PKCγ control of gap junctions for maintenance of Purkinje cells.

2213/B684

The Protective Effect of Endoplasmic Reticulum Chaperone Protein Grp78 Is Associated with Its Retargeting to Mitochondria and Maintaining Mitochondrial Function Following Injury in Astrocytes.

Y. Ouyang, L. Xu, J. Emery, R. G. Giffard; Anesthesia, Stanford University, Stanford, CA

Grp78 (also called Hsp78 or Bip) is an ER-resident molecular chaperone and a member of the heat shock protein 70 family. We have shown that over-expression of endoplasmic reticulum chaperone protein Grp78 protects astrocytes from ischemia- like Injury. Here we further investigate the mechanism of the protection involved. Immunostaining for Grp78 in control unstressed cells revealed a purely perinuclear distribution, consistent with ER localization, and showed little overlap with a mitochondrial label, mitotracker. After glucose deprivation (GD), staining for Grp78 was seen much more extensively throughout the cell in a more vesicular pattern and now extensively colocalizing with mitotracker. It is interesting that in Grp78 over-expressing cells, achieved with transfection with an expression plasmid, this Grp78 retargeting to mitochondria happened as early as 1 hr after initiation of GD compared to 5 hr of GD in the control injury group. The redistribution of Grp78 in cells induced by GD observed morphologically by immunostaining was confirmed by cellular fractionation and Western blotting with antibodies specific for Grp78. The function of Grp78 in mitochondria has not been reported before and we find here that it attenuates the increase of mitochondrial calcium and free radicals as well as preserves mitochondrial membrane potential and its electron respiration chain complex activity. Thus we observe Grp78 in mitochondria following stress which is associated with improved mitochondrial function and improved cell survival.

2214/B685

The Early-Onset Torsion Dystonia Gene Product, Torsina, Is a Mediator of Intracellular Stress and Homeostasis.

P. Chen, J. C. Ricketts, A. J. Burdette, L. A. Berkowitz, K. A. Caldwell, G. A. Caldwell; Biological Sciences, The University of Alabama, Tuscaloosa, AL

The capacity of cells to carry out their various functions is wholly dependent upon efficient protein synthesis, processing, trafficking, and degradation. In this context, dysfunction or deficit of proteins that effectively monitor and ensure the proper maintenance of cellular homeostasis can have serious consequences in terms of disease onset, penetrance, or progression. A single codon deletion (ΔE) in the gene encoding human torsinA causes a non-degenerative neurological disorder termed early-onset torsion dystonia. TorsinA, an endoplasmic reticulum (ER) resident protein, belongs to the diverse AAA+ (ATPases Associated with a variety of cellular Activities) family. Here we use a well established ER stress model in the nematode *C. elegans* (*hsp-4*::GFP) to investigate the role of torsinA in maintaining protein homeostasis in the ER. Using GFP as an ER stress reporter, we found that transgenic nematodes expressing wild type (WT) human torsinA exhibited a striking reduction in ER stress caused by tunicamycin, while animals with either ΔE or a mixture of WT/ΔE torsinA failed to do

so. Interestingly, ΔE and WT/ΔE torsinA worms exhibited higher ER stress than WT torsinA animals, even in the absence of tunicamycin treatment. In addition, mutations within torsinA ER signal sequence and N-terminal hydrophobic region abolished its capacity to suppress ER stress, indicating the importance of its localization to the ER for this activity. Furthermore, although torsinA possesses very low ATPase activity, the ATPase domain appears important for ER stress suppression, as two mutations within the ATPase domain, K108A (causing defect in ATP binding) and E171Q (blocking ATP hydrolysis), resulted in loss of ER stress suppression. Moreover, human torsinB, with 70% homology to torsinA, could not suppress ER stress after tunicamycin treatment and even caused stress by itself, when overexpressed. Taken together, our data indicate that torsinA helps to maintain protein homeostasis in the ER, shedding new light on the pathophysiology of torsion dystonia.

2215/B686

Dystonia-Associated Mutations Cause Premature Degradation of Torsina Protein and Cell Type-Specific Mislocalization to the Nuclear Envelope.

L. Giles, J. Chen, L. Li, L. Chin; Department of Pharmacology, Emory University School of Medicine, Atlanta, GA

An in-frame 3-bp deletion in the torsinA gene resulting in the loss of a glutamate residue at position 302 or 303 (torsinA ΔE) is the major cause for early-onset torsion dystonia (DYT1). In addition, an 18-bp deletion in the torsinA gene resulting in the loss of residues 323-328 (torsinA Δ323-8) has also been associated with dystonia. Here we report that torsinA ΔE and torsinA Δ323-8 mutations cause neuronal cell type-specific mislocalization of torsinA protein to the nuclear envelope without affecting torsinA oligomerization. Furthermore, both dystonia-associated mutations destabilize torsinA protein in dopaminergic cells. We find that wild-type torsinA protein is degraded primarily through the macroautophagy-lysosome pathway. In contrast, torsinA ΔE and torsinA Δ323-8 mutant proteins are degraded by both the proteasome and macroautophagy-lysosome pathways. Our findings suggest that torsinA mutation-induced premature degradation may contribute to the pathogenesis of dystonia via a loss-of-function mechanism, and underscore the importance of both the proteasome and macroautophagy in the clearance of dystonia-associated torsinA mutant proteins.

2216/B687

Generation of Analog Sensitive-Pkcδ to Isolate Direct Substrates of Pkcδ in Stroke-Reperfusion Injury.

W. Chou[1], M. Rosen[1], D. Wang[1], J. J. Allen[2], K. Shokat[2], R. O. Messing[1]; [1]Neurology, Gallo Center, UCSF, Emeryville, CA, [2]Cellular and Molecular Pharmacology, HHMI, UCSF, San Francisco, CA

Thrombolytic agents such as tissue plasminogen activator (t-PA) are currently the only drugs available to reverse acute ischemic stroke, but paradoxically reestablishment of circulation may initiate reperfusion injury. Therefore, there is great interest in developing treatments to limit reperfusion injury. Studies with mouse genetics, experimental stroke models, and selective peptide inhibitors and activators have implicated PKCδ in stroke-reperfusion injury, in particular PKCδ in neutrophils. Understanding the molecular and cellular actions of PKCδ in reperfusion injury requires the identification of the unique targets of PKCδ in signaling pathways activated by ischemia and reperfusion. We are using a novel chemical-genetics approach to identify immediate substrates of kinases. Based on homology with other analog-sensitive kinase mutants, we generated and characterized an analog sensitive-PKCδ mutant (as-PKCδ) that is sensitive to analogs of ATP and the kinase inhibitor PP1. We expressed PKCδ wild type (WT) and as-PKCδ mutant kinases in COS-7 and Neuro2A cells, and found that as-PKCδ showed levels of expression near WT PKCδ when transfected into both cell types. In vitro kinase assays showed that unlike WT PKCδ, the as-PKCδ mutant can utilize an ATP analog (N6-(benzyl)-ATP) to phosphorylate a model PKC substrate (histone III) in vitro, and is uniquely inhibited by the novel PP1 analog 1-Naphthyl-PP1 (1Na). Recently we succeeded in generating knock-in mice expressing as-PKCδ. Using neutrophils isolated from these knock-in mice, we identified several potential PKCδ substrates phosphorylated in the presence of N6-(benzyl)-ATP-γS. We purified some of these putative PKCδ substrates by isolectric focusing fractionation and SDS-PAGE. The identities of these substrates were revealed by mass spectrometry and highlight potential signaling pathways in which PKCδ participates during stroke-reperfusion injury.

2217/B688

Functional Analysis of PKCδ Gene Promoter in Neuronal Cells: Implications of Oxidative Damage in Dopaminergic Neurodegeneration.

H. Jin, A. Kanthasamy, V. Anantharam, A. Kanthasamy; Biomedical Sciences, Iowa State University, Ames, IA

Protein kinase Cδ (PKCδ) is an oxidative stress sensitive kinase known to play a causal role in apoptotic cell death in Parkinson's disease models. We recently demonstrated that a minimal 148 bp promoter region drives basal PKCδ transcription. To further characterize the regulation of PKCδ gene expression, we cloned an approximately 2k-bp segment of the 5' flanking region of the mouse PKCδ gene, which contains non-coding exon1 regulatory elements. Deletion analysis and in silico promoter examination revealed multiple Sp sites, including four GC boxes and one CACCC box, in the first, non-coding exon1, significantly enhance basal transcription from the minimal promoter sequence in NIE115 and MN9D cells. Detailed site-directed mutagenesis revealed that each identified Sp binding site made a positive contribution to PKCδ promoter activity. Functionally, overexpression of Sp1, Sp3, and Sp4 markedly stimulated PKCδ promoter activity without any synergistic transactivating effect. Furthermore, in Drosophila Schneider cells lacking endogenous Sp factors, Sp3 overexpression potently up-regulated the PKCδ promoter by ~100-fold, indicating Sp3 is the essential activator of PKCδ transcription. Conversely, blockade of Sp binding with mithramycin A inhibited PKCδ promoter activity in a dose-dependent manner. The incubation of nuclear extracts with fluorescence-labeled oligonulceotide containing the two proximal GC boxes gave a single gel-shifted complex that was blocked by its respective cold or Sp-1 consensus oligonucleotides, but not by its mutant oligonucleotides. The results from chromatin immunoprecipitation assay further confirmed that Sp3, and less significantly, Sp4 and Sp1, binds to PKCδ promoter. Taken together, these data indicate that Sp transcription sites, in particular Sp3, play a pivotal role in the regulation of PKCδ basal transcription, which may have implications for development of new therapeutic strategies against PD.

2218/B689

Lymphocyte Subpopulations in Children with Severe Neurological Disorders Caused by Perinatal Hypoxic-Ischemic CNS Damage.

T. Mardovina; Mother and Child, Minsk, Belarus

Objective: Under supervision there were 36 children of the 1-2 years old with severe neurological disorders caused by perinatal hypoxic-ischemic CNS damage (cerebral palsy, delay of motor development, hypertension syndrome) in National Scientist Practical Center "Mother and Child" (Minsk, Belarus). The aim of the present study was to evaluate lymphocyte subpopulations: CD3+, CD4+, CD3+CD8+, CD19+, CD3+CD56+, CD3-CD56+, CD3+CD25+, CD3-CD25+, CD3+HLA DR+,CD3-HLA DR. Methods: multiparameter flow cytometry The following combination of mouse antihuman moAbs were used for five color staining in 2 panels: 1 panel: CD4-FITC/CD8-PE/CD3-PC5/CD45-ECD/CD56-PC7 2 panel: CD25-FITC/CD3-PC5/CD19-PC7/HLA-DR-PE/CD45-ECD Data were analyzed by FACScan flow cytometer (Beckman Coulter). Results: We founded decreasing of the number T- helpers (35,3 ± 2,48) p<0,05; increasing of the number of B-lymphocytes (31,6± 1,45) p<0,05, increasing CD3+HLA DR+ (1,8± 0,24)) p<0,05, CD3-HLA DR+ (32,2±1,48) p<0,05. From ANAMNES: All children had acute viral diseases of respiratory tracts (pharyngitis, tracheitis, bronchitis) 3-4 cases in a year. CONCLUSIONS: Children with severe neurological pathology caused by perinatal hypoxic-ischemic CNS damage have Immunity disorders and require dynamic supervision by immunologyst.

2219/B690

Elucidating the Role of RAGE Signaling in Neuronal Regeneration in the Diabetic System.

J. Z. Liu[1], R. H. Rosario[1], E. Boulanger[1], A. M. Schmidt[1], M. S. Geddis[2,1]; [1]Surgery, Columbia University, New York, NY, [2]Science, BMCC-CUNY, New York, NY

Activation of the Receptor for Advanced Glycation Endproducts (RAGE) has been reported to be involved in neuronal regeneration after *In Vivo* peripheral nerve injury. The precise role of RAGE in neuronal regeneration is complicated not only by its deleterious effects in other diabetic systems but also by its expression on multiple components of the regeneration scheme, most notably neurons and macrophages. In this series of experiments, isolated dorsal root ganglion (DRG) neurons from 3-week old wild type (WT) and RAGE-/- mice were plated on collagen-coated tissue culture dishes and observed for key indicators of regeneration. RAGE activation was induced by the ligands, carboxymethyl-lysine-advanced glycation end product (CML-AGEs) and S100b to simulate hyperglycemic and euglycemic conditions. Neuronal survival studies which determined the number of neurons alive over time indicated that exposure of WT neurons to S100b (n=8, 10 µg/ml) and CML-AGEs (n=4, 10 µg/ml) increased survival, 22.8 ± 7.6% and 6.8 ± 6.0%, respectively, at day 3 but decreased survival 35.6 ± 7.8% and 9.3 ± 6.5% at day 7 as compared to controls (n=4) suggesting that RAGE signaling may play a positive acute role but a negative long-term role in neuronal survival. Furthermore, the number of neurites formed on WT neurons in the presence of S100b and CML-AGEs was increased at day 3 by 237.5 ± 15.6% (n=5) and 185.2 ± 66.0% as compared to controls (n=5). Neurons were also observed for neurite outgrowth over a time-course of 1 hour before and after exposure to ligands in an environmental chamber. As compared to controls (n=3), WT neuronal outgrowth rates 1 hour after s100b (n=4) exposure decreased 47.1 ± 17.1% as neurites retracted. Ligand exposure to DRGs isolated from RAGE-/- mice, did not significantly alter any of the regeneration parameters. Overall, measurement of these 3 indices revealed that exposure to RAGE ligands *in vitro* is at first beneficial and then detrimental, suggesting that RAGE has a complex role in neuronal regeneration. Supported by: NIH grant AG17490.

2220/B691

Plasma Progranulin Levels Predict Progranulin Mutation Status in Frontotemporal Dementia Patients and At-Risk Individuals.

N. Finch[1], M. Baker[1], R. Crook[1], M. Heckman[1], K. Kuntz[2], D. Dickson[1], B. Boeve[2], N. Graff-Radford[1], R. Rademakers[1]; [1]Neuroscience, Mayo Clinic, Jacksonville, FL, [2]Neurology, Mayo Clinic, Jacksonville, FL

Objective: To determine whether a plasma progranulin (GRN) enzyme-linked immunosorbent assay (ELISA) can be used to predict GRN mutation status. Material & Methods: GRN sequencing analyses in a sequential series of clinical FTLD patients (N=188) ascertained at a Behavioral Neurology referral practice. Onset age was 63.3 ± 8.3 years (range 44-81 years) with a family history of dementia in 77 patients (41%). Plasma GRN ELISA was performed in duplicate in 175 patients with plasma availability. Results: GRN loss-of-function mutations were identified in 7 patients (3.7% of population), including 2 apparently sporadic patients. Mutations included the common p.R493X nonsense mutation, frameshift mutations resulting from small insertions or deletions and the p.A9D mutation located in the GRN signal peptide. We also identified a potential pathogenic missense mutation, p.C139R, in an 80-year old patient with a 4-year history of SD, which was absent in 200 controls. p.C139R affects a highly conserved cysteine residue in the cysteinyl motif of granulin F, which might disrupt the disulfide bridges in GRN leading to a loss-of-function. Using a GRN ELISA, we demonstrate that plasma GRN levels are significantly reduced by ~3-fold in all patients carrying GRN loss-of-function mutations compared to non-GRN carriers (p<0.001). GRN levels ranged from 53-77ng/ml (average 60 ± 9ng/ml) in mutation carriers, while non-GRN carriers showed levels from 102-328ng/ml (176 ± 39ng/ml). The GRN level in the p.C139R mutation carrier was among the 5% lowest of all patients (105ng/ml), though outside the range of typical loss-of-function mutations, suggesting that p.C139R may induce a partial loss-of-function. Interestingly, significantly reduced GRN levels (81ng/ml) were also observed in plasma of a 62-year old asymptomatic at-risk individual carrying a loss-of-function GRN mutation. Analysis of additional asymptomatic at risk individuals is in progress. Conclusions: Plasma GRN levels can predict GRN mutation status in FTLD patients and asymptomatic at-risk individuals.

2221/B692

Investigating the Role of Histone Deacetylases in C. Elegans Neurotransmission.

B. B. Kautu[1], K. Berry[1], C. Locke[1,2], S. Lee[1], G. Caldwell[1], K. A. Caldwell[1]; [1]Biological Sciences, University of Alabama, Tuscaloosa, AL, [2]University of California-San Francisco, San Francisco, CA

Histone deacetylases (HDACs) are a class of enzymes that repress gene expression through posttranslational modification of histones. Studies in *C. elegans* have illuminated the developmental roles of HDACs in vulval development, as well as axon migration and pathfinding. Despite the established role of HDACs in development, little is known about the functional contribution of HDACs in the *C. elegans* nervous

system. Here, we investigated the role of HDACs in synaptic transmission, using a combination of genetic and pharmacological approaches. Depletion of the *C. elegans* ortholog of *hda-1*, the human HDAC1, by lactose-induced RNAi feeding conferred hypersensitivity to aldicarb, an inhibitor of acetylcholinesterase, suggesting that *hda-1* may regulate synaptic transmission at the *C. elegans* NMJ. Alternatively, knockdown of other worm HDAC genes, including *hda-2*, *hda-3*, *hda-4*, and F41H10.6, failed to confer hypersensitivity to aldicarb. In attempting to further discern putative targets of HDAC-mediated regulation, we examined specific gene products that we have previously shown to impact synaptic transmission in *C. elegans*. We found that knockdown of *hda-1* in both *ina-1* (integrin) loss-of-function and *mig-2* (Rac GTPase) gain-of-function mutants enhanced sensitivity to aldicarb synergistically. Likewise, depletion of *hda-4* in either an *ina-1* or *mig-2* mutant background revealed synergistic interactions. Interestingly, depletion of the *epi-1* gene, a *C. elegans* ortholog of the human laminin gene, which is implicated in human epilepsy, conferred hypersensitivity to aldicarb (and previous studies showed that *epi-1* genetically interacts with *hda-1* in *C. elegans*). Depletion of *epi-1* in both *ina-1* and *mig-2* mutants synergistically enhanced sensitivity of *ina-1* and *mig-2* mutants to aldicarb. Taken together, these data suggest that *hda-1* and *hda-4* may function in a common pathway with integrin, laminin, and a Rac GTPase to modulate post-embryonic synaptic transmission. Examination of changes in integrin, laminin, and Rac GTPase gene expression, as well as on synaptic vesicle distribution and neuronal architecture, and will serve to further define the contribution of specific HDACs in neurotransmission.

2222/B693

Anatomic and Molecular Changes in Retinitis Pigmentosa.

R. Mullins[1], S. Enriquez[1], E. Faidley[1], E. Schindler[1], M. Kuehn[1], E. Stone[1,2]; [1]University of Iowa, Iowa City, IA, [2]Howard Hughes Medical Institute, Iowa City, IA

Objective: Retinitis pigmentosa (RP) is a rare hereditary form of retinal degeneration. This study was performed to evaluate the morphological and gene expression features of advanced autosomal recessive RP in human retinal tissue. Methods: Eyes were obtained from a donor with a clinical history of autosomal recessive RP. Retinal tissue was either preserved for histological processing or was used for RNA isolation. Immunohistochemical labeling with antibodies directed against glial, neuronal and vascular markers was performed and labeling was compared to results from a set of unaffected donor eyes. Expression analysis was performed using Affymetrix expression arrays and results were compared with a sample from an unaffected eye. Results: Histological anlaysis revealed complete degeneration of photoreceptor cells with loss of an identifiable outer nuclear layer and disruption of the retina, migration of the retinal pigment epithelium (RPE) and a small region of neovascularization and scarring. Loss of the RPE and choriocapillaris was regional, with some areas showing preservation and others showing complete atrophy. Increased expression and redistribution of the glial marker GFAP was observed. Gene expression analysis revealed expected loss of photoreceptor cell specific transcripts, such as rhodopsin. Interestingly, many transcripts associated with inner retinal neurons, such as the genes encoding the rod bipolar cell glutamate receptor (Grm6) and neurofilament heavy chains (NEFH), were still present. Expression of GFAP transcripts was increased, consistent with immunohistochemical observations. Conclusions: To our knowledge this is the first report of global gene expression analysis in human retinitis pigmentosa. The persistence of bipolar and ganglion cell transcripts suggests that, in spite of the significant plasticity that occurs during retinal degeneration, bipolar cells and ganglion cells remain at least partially differentiated. This study suggests that experimental therapies may have benefit in advanced retinal degeneration.

2223/B694

Natural Polyphenols Attenuate Lipopolysaccharide-Induced Neuroinflammation in BV-2 Microglia Cells and Dopaminergic Neuronal Cell Death.

E. Park, H. Chun; biotechnology, Chosun Univ., Gwang ju, South Korea

Parkinson's disease (PD) is a common, progressive neurodegenerative disorder caused by the loss of dopaminergic neurons in the substantia nigra. Neuroinflammation plays a role in the pathophysiology of many neurodegenerative diseases including PD. Previous studies suggested that lipopolysaccharide (LPS) induced the release of nitric oxide (NO) and proinflammatory molecules such as tumor necrosis factor-alpha (TNF-a), interleukin-1 beta (IL-1b) and interleukin-6 (IL-6). In this study, we have examined the effect of BV-2 (microglial cell) conditioned media which was activated with LPS. Caspase-3 was markedly activated and apoptotic cell death was increased by BV-2 conditioned media in dopaminergic neurons. However, apoptosis was mitigated by pretreatment with natural polyphenols such as resveratrol (500nM) and EGCG (2uM). These results suggest that natural polyphenols may inhibit the neuroinflammation and subsequent dopaminergic neuronal cell death. [Supported by BK21 program of MOE and the SRC/ERC program of MOST/KOSEF(R11-2000-083-02006-0, Korea]

2224/B695

The Effects of Rhus Verniciflua Stokes on Brain Aging Model in Mice Induced by D-Galactose and Nano2.

H. Jung[1], H. Go[2], C. Jeong[3], J. Park[3], H. Yim[1], S. Ko[1]; [1]Kyunghee University, Seoul, South Korea, [2]Semyeong University, Chungbuk, South Korea, [3]Kyungwon University, Seongnam, South Korea

Objective: The aim of this study was to investigate the neuroprotective effects of Rhus verniciflua Stokes on brain aging mouse model induced D-galactose and NaNO2. Methods: To make brain aging of mice, control and experimental group mice were intraperitoneal injected D-galactose 120mg/kg, NaNO2 90mg/kg once daily for 60 days. From day 47, control group mice were p.o. administered with saline 0.3ml for each, and experimental group mice were p.o. administration Rhus verniciflua Stokes. Normal group mice were intraperitoneal injected saline 0.3ml for 60 days and p.o. administration saline 0.3ml each mouse from 47th day. To investigate neuropathological changes, inflammation, apoptosis, and antioxidant effects on brain aging tissue, it is used cresyl violet staining method, immunohistochemical staining with OX-42, Bcl-2, Bax and measured contents of malondialdehyde and the activities of superoxide dismutase. Resuls and Conclusions: The results indicated that combined injection of D-galactose and NaNO2 induced brain aging of mice and activities of superoxide dismutase activity decreased and malondialdehyde level increased in mouse brain. Administration of Rhus verniciflua Stokes for 14 days significantly improved the biochemical marker mentioned above and reduced the histological lesion in mouse brain. These results suggests that Rhus verniciflua Stokes is worth testing for future preclinical study aimed for brain aging and neurodegenerative diseases.

2225/B696

Differential Effects of Scutellaria, Baicalin and Baicalein on Inflammatory Response in Lipopolysaccharide-Induced BV2 Microglial Cells.

Y. Yang[1], G. Kim[2], C. Jeon[2], S. Sun[3], H. Yim[1], S. Ko[1]; [1]Kyunghee University, Seoul, South Korea, [2]Kyungwon University, Seoul, South Korea, [3]Sangji University, Wonju, Kangwon-Do, South Korea

Objective: The root of Scutellaria is a traditional medicine that is commonly used for the treatment of hepatitis, tumors, diarrhea and inflammatory diseases. The major chemical constituents of Scutellaria are flavonoids. Baicalin and Baicalein are flavonoids found in the root of Scutellaria baicalensis Georgi that have previously been shown to have anti-inflammatory activities in various cell types, including microglial cells. The aim of this study was to investigate the neuroprotective effects of Scutellaria baicalensis on brain aging mouse model. Methods: In this study, the effect of Scutellaria, Baicalin and Baicalein extract on Lipopolysaccharide-induced inflammation in mouse BV2 microglial cells was investigated using an MTS assay, RT-PCR, Western blot and HPLC. Results: The results of this study revealed that Scutellaria, Baicalin and Baicalein extract suppressed nitric oxide production by inhibiting the Lipopolysaccharide-Stimulated enhancement of COX-2 and iκNOS expression. Moreover, Scutellaria, Baicalin and Baicalein were found to downregulate several of the Lipopolysaccharide-induced signaling molecules, including P38, ERK1/2 and the JNK mitogen-activated protein kinase (MAPK) pathway. In addition, Scutellaria, Baicalin and Baicalein were found to suppress NF-κB activation by blocking IκBα degradation, which is involved in the regulation of inflammatory responses. Conclusions: Taken together, these data show that Scutellaria, Baicalin and Baicalein may be useful for the treatment of inflammatory diseases. In addition, the results of HPLC analysis revealed that Scutellaria inhibited LPS-induced iNOS (but not COX-2) protein and gene expression to a greater degree than a mixture of Baicalin and Baicalein, and that treatment with a mixture of Baicalin and Baicalein resulted in greater inhibition of iNOS expression than treatment with either compound alone. Keywords: Scutellaria, Baicalin, Baicalein, lipopolysaccharide, HPLC

2226/B697

The Heparan Sulphate Proteoglycan, Glypican-1, Mediates Both Normal Prion Protein Lipid Raft Association and Disease Isoform Formation.

D. R. Taylor, I. J. Morten, N. M. Hooper; Institute of Molecular and Cellular Biology, Faculty of Biological Sciences and Leeds Institute of Genetics, Health and Therapeutics, University of Leeds, Leeds, United Kingdom

The prion protein (PrP) is the causative agent of the prion diseases. In these diseases, the normal cellular form of PrP (PrP^{C}) undergoes a conformational conversion to the infectious scrapie isoform, PrP^{Sc}. Many aspects of prion disease are well characterised yet the subcellular site of prion conversion remains controversial. After synthesis, PrP^{C} resides in cholesterol-rich lipid raft domains on the cell surface, from which it dissociates to be endocytosed via clathrin-coated pits. Both lipid rafts and the endocytic pathway are suggested sites of PrP^{Sc} formation, highlighting the importance of understanding PrP's membrane trafficking in detail. Whilst PrP^{C}'s association with lipid rafts is due, in part, to its glycosyl-phosphatidylinositol (GPI) anchor, the N-terminal domain contains additional raft targeting determinants. This domain binds to heparan sulphate, a component of heparan sulphate proteoglycans (HSPGs). Significantly, HSPGs have been implicated as both receptors for PrP^{Sc} and as co-factors in PrP conversion. Accordingly, the aim of this study was to investigate whether HSPG species mediate membrane trafficking and lipid raft association of PrP^{C} and its conversion into PrP^{Sc}. To determine the identity of proteins targeting PrP to lipid rafts a transmembrane-anchored mutant of PrP (PrP-CTM) was studied in SH-SY5Y human neuroblastoma cells. This mutant associates with rafts via its N-terminal domain only. PrP-CTM raft association was abrogated by addition of exogenous heparin or phosphatidylinositol-specific phospholipase C treatment, suggesting a GPI-anchored HSPG targets PrP to lipid rafts. Glypican-1 (GPC1) is the major neuronal GPI-anchored HSPG. GPC1 depletion by siRNA significantly reduced PrP-CTM raft association, implying GPC1 is a raft binding partner of PrP. Basal endocytosis of PrP^{C} was enhanced with GPC1 depletion suggesting GPC1 regulates PrP^{C} membrane trafficking. N2a mouse neuroblastoma cells chronically infected with PrP^{Sc} were treated with GPC1 siRNA to study its role in prion conversion. Depletion significantly reduced the load of PrP^{Sc} in these cells. In summary the data presented identify GPC1 as a lipid raft binding partner of PrP^{C} and a novel co-factor in prion conversion.

2227/B698

Assessing Profilin IIa Expression in an SMA Mouse Model.

M. Bowerman[1,2], R. Kothary[1,2]; [1]Ottawa Health Research Institute, Ottawa, ON, Canada, [2]Cellular and Molecular Medecine, University of Ottawa, Ottawa, ON, Canada

Spinal muscular atrophy (SMA) is the most common genetic disease resulting in infant mortality. SMA manifests itself due to a severe loss of α-motor neurons and is caused by mutations or deletions of the ubiquitously expressed survival motor neuron 1 (SMN1) gene. However, why α-motor neurons of SMA patients are specifically affected is not clear. We have previously demonstrated that Smn-deficient PC12 cells show an increase in the neuronal-specific profilin IIa (pIIa) isoform. Moreover, the depletion of Smn, a known interacting partner of pIIa, further contributes to the increased pIIa availability. We therefore suggest that this leads to altered cytoskeletal integrity and a subsequent defect in neuritogenesis. We are presently investigating the expression of the profilin IIa isoform in an SMA mouse model developed in our laboratory, termed Smn2B/-. This mouse model is characterized by a loss of α-motor neurons in the spinal cord and brain stem at P21 and a reduced life expectancy when compared to littermates. Here, we show that there is an increase in pIIa signal intensity in neuronal cells within the spinal cord of Smn2B/- mice. Whole mount in situs will be performed to determine if this is due to increased protein stability or increased gene transcription. Furthermore, crosses with the profilin II (pfn2) knock-out mouse are underway and will allow us to determine if heterozygous and/or homozygous profilin II reduction rescues the SMA phenotype in our Smn2B/- mouse model.

2228/B699

Involvement of TRPA1 in Hydrogen Peroxide Induced Pain.

H. Hosokawa, Y. Sawada, S. Tsujikawa, S. Maegawa, K. Matsumura, S. Kobayashi; Kyoto University, Kyoto, Japan

Hydrogen peroxide (H2O2) contained in industrial products is also generated within the cells. H2O2 causes pain, but it has not been elucidated how H2O2 activates sensory neurons in the pain pathway. Here we show that Transient Receptor Potential Ankyrin-1 (TRPA1), expressed by sensory neurons in the pain pathway, is a receptor for H2O2. H2O2 activated TRPA1 to induce Ca2+ influx and elicit non-selective cation currents. These effects of H2O2 were mimicked by both reactive oxygen species and reactive nitrogen species. Cysteine reducing agents suppressed H2O2-induced TRPA1 activation whereas cysteine oxidizing agents activated TRPA1. H2O2 caused Ca2+ influx in a subset of dorsal root ganglia (DRG) neurons which also responded to a chemically TRPA1 ligand. These results suggest that TRPA1 plays a key role for sensation of pain caused by H2O2.

2229/B700

Aberrant Intracellular Calcium Dynamics of Neurohypophysis of Thalidomide-induced Autistic Rats.

N. Ishikita[1], A. D. Russa[1,2], K. Masu[1], T. KURODA[1], H. Akutsu[1], T. Saino[1], Y. Satoh[1]; [1]Anatomy (Cell Biology), Iwate-Medical University, Morioka, Japan, [2]Anatomy & Histology, Muhimbili University of Health and Allied Sicences, Dar es Salaam, Tanzania

[Objective] Autism is a psychological development disorder characterized by social impairment, communication deficits, and compulsive behavior. Although current understanding about the etiology of the disease is limited, recent investigation suggested that neuro-hormonal disorders (e.g., low level of oxytosin) may play a role in developing autism. The present study aimed to assess whether disorder of intracellular calcium ([Ca2+]i) signaling mechanism which is an essential signaling in various function including secretory activity in nervous system occur in autistic conditions. To this end, we used thalidomide-induced autistic rat neurohypophysis, as a specimens for [Ca2+]i monitoring. **[Methods]** We administered 500 mg/kg thalidomide orally to pregnant wistar rats (E9). The male pups were sacrificed, and hypophysis were cut into 150 µm in thick by microslicer. The slice specimens were loaded with Indo-1 (a Ca2+ indicator) and spatio-temporal [Ca2+]i dynamics during membrane depolarization were examined by a real-time confocal microscope. Under the fluorescence imaging, we could easily distinguish between adeno- and neurohypophysis. **[Results]** In neurohypophysis, nerve endings and glia cells (pituitocytes) were distinguished under the fluorescent microscopy. When the control pituitary was stimulated for 1 min by high K+, [Ca2+]i of both nerve endings and pituitocytes increased, but the dynamics of nerve ending was prominent. The increase of nerve endings was rapid and then declined gradually. In most cases, after the initial increase, moderate increase was kept. These plateau of [Ca2+]i increase was resulted by Ca2+ replenishment from Ca2+ influx, and it may useful to enhance the secretory activities. In thalidomide-treated rat, neurohypophysis showed weaker responses, and faster decrease. The differences was statistically verified. Results based on extracellular Ca2+ free, Gd or thapsigargin treatment examination indicate the abnormality of store-operated calcium channels (SOC) in thalidomide-treated autistic rats. **[Conclusion]** When some dysfunctions of SOC of neurohormonal cells occur, oxytosin secretion inhibited, and the caused behavial disorder; autism.

Host-Pathogen Interactions: Viruses (2230 – 2245)

2230/B701

Live Cell Imaging of HIV-1-Host Protein-protein and RNA-protein Interactions by Tri- and Bi-fluorescence Complementation.

M. Milev[1,2], A. Mouland[1,2]; [1]HIV-1 RNA Trafficking Laboratory, Lady Davis Institute for Medical Research, Montreal, QC, Canada, [2]Experimental Medicine, McGill University, Montreal, QC, Canada

As an obligate intracellular parasite, human immunodeficiency virus type 1 (HIV-1) uses cellular proteins and machineries to ensure transmission to uninfected cells. One of the late events of the HIV-1 replication is the trafficking of the viral genomic RNA (vRNA). This step involves the nuclear export of the vRNA, the formation of cytoplasmic ribonucleoproteins (RNPs) and vRNA trafficking to sites of viral assembly. We have shown that the dsRNA-binding protein, Staufen1 is found in the context of a RNP containing the vRNA and the viral structural protein, Gag (HIV-1 RNP). Other RNA binding proteins were found in the HIV-1 RNP using tandem affinity purification followed by mass spectrometry such as insulin-like growth factor II mRNA-binding protein (IMP1) and further characterized. We investigated the possible interactions between Gag, Staufen1 and IMP1 with RNA reporter molecules in vivo. Using bimolecular and trimolecular fluorescence complementation techniques (BiFC and TriFC) that allow for direct visualization and localization of protein-protein and RNA interactions in living cells, we show that Gag & Staufen1 and Staufen1 & IMP1 interact, as demonstrated by the appearance of fluorescent punctae in the cytoplasm. IMP1 and Gag were also found to interact in vivo. Furthermore, these interactions occurred when one of the partners was tethered to mRNA. The granules were found to be dynamic structures such that several fluorescent punctae would appear or extinguish over time. Also, the RNPs were mobile as judged by time lapse photography and had a trajectory consistent with the notion that the HIV-1 RNP traffics outbound towards the cell periphery and this movement was dependent on Staufen1 expression. This work provides new information on the composition and the formation of HIV-1 RNPs during the assembly of infectious particles.

2231/B702

HIV-1 Nef Mediates Ubiquitination-Independent Targeting of CD4 to the MVB Pathway.

L. L. DaSilva, R. Sougrat, P. V. Burgos, K. Janvier, J. Bonifacino; CBMP, NICHD/NIH, Bethesda, MD

The HIV-1 accessory protein Nef plays essential roles in viral replication and progression to AIDS. Among the functions of Nef is the downregulation of the co-receptor, CD4. To achieve this, Nef induces CD4 internalization from the cell surface leading to its degradation in lysosomes. Internalization of CD4 by Nef is known to depend on clathrin and AP-2, but the later steps of CD4 downregulation are still poorly understood. We have found that, in the presence of Nef, internalized CD4 colocalizes with components of the MVB machinery and is targeted to intralumenal vesicles of MVBs. Moreover, CD4 degradation in lysosomes requires functional Endosomal Sorting Complex Required for Transport (ESCRT) components. Although CD4 undergoes ubiquitination, this modification is not required for either its internalization or lysosomal targeting induced by Nef. Nef is likewise ubiquitinated, but this modification is also dispensable for CD4 downregulation. Nef and CD4 colocalize to endosomal structures, but in contrast to CD4, Nef itself appears to escape lysosomal degradation.

In conclusion, our findings indicate that besides accelerating endocytosis, Nef has the function to target CD4 to MVBs in a ubiquitination-independent fashion.

2232/B703

Novel Antifungal Effects of Rev-NIS Derived from HIV-1 Regulatory Protein, Rev.

D. Lee, J. Lee; School of Life Sciences & Biotechnology, College of Natural Sciences, Kyungpook National University, Daegu, South Korea

Rev is a small regulatory protein of HIV-1 essential for virus replication. Especially, Rev (11-20) is known for an essential region of nuclear entry inhibitory signal (Rev-NIS, ELLKAVRLIK) and the conserved hydrophilicity motif, which forms an amphipathic helix. In the present study, we investigated novel antifungal activities and its mode of action of Rev-NIS. Rev-NIS had potent antifungal activities against human pathogenic fungal strains without remarkable hemolytic effect towards human erythrocytes. To investigate the mechanism(s) of Rev-NIS, flow cytometry analyses were performed by using bis-(1,3-dibutylbarbituric acid) trimethine oxonol, and changes in membrane dynamics were assessed by using 1,6-diphenyl-1,3,5-hexatriene. The results suggest that Rev-NIS may exert its antifungal effect by disrupting the cell membranes. These results were further confirmed by using calcein leakage measurement with liposome mimicking fungal membranes. The current study suggests that Rev-NIS may have potential as novel peptidic therapeutic agents for human fungal infectious diseases.

2233/B704

From Target Identification to Drug Discovery: the HIV-1 Integrase / Human LEDGF Case Study.

E. Vinolo[1], P. Tafelmeyer[1], P. Guedat[1], J. Rain[1], S. Emiliani[2], E. Formstecher[1], F. Moisant[1], A. Pecquery-Texier[1], S. Barbey[3], J. Paris[3], R. Benarous[3]; [1]HYBRIGENICS SA Services, Paris, France, [2]Institut Cochin, Paris, France, [3]CellVir SA, Evry, France

More than 25 years after its discovery, Human Immunodeficiency Virus 1 (HIV-1) is still one of the major threads of human life. Most anti-HIV drugs currently in use inhibit the catalytic activities of viral enzymes. However, novel mechanisms of action are needed to cope with the increasing resistance against these drugs. A promising alternative is to target the complex interplay between viral proteins and host cells. We used Hybrigenics' ULTImate Y2H™ technology to perform a large-scale yeast two-hybrid study with all HIV-1 proteins as baits, using highly complex cDNA libraries from the CEMC7 T cell line. Analysis of the interaction network led to the selection and functional validation of several protein interactions. We will detail the in-depth characterization and chemical targeting of the interaction between HIV-1 integrase and the human transcriptional co-activator LEGDF (1). siRNA knockdown of LEDGF demonstrated the functional importance of this interaction for targeting integrase to the chromosome. Using a random-primed HIV-1 library, the integrase catalytic core was mapped as the smallest interacting domain in a rebound yeast two-hybrid screen with LEDGF as bait. Moreover, by screening loss-of-affinity mutants in yeast, Q168 of integrase was identified as one of the hot-spots of the interaction. Based on these functional and detailed molecular evidences, we decided to target the HIV-1 integrase / human LEDGF interaction to identify new molecules with therapeutic use in HIV treatment. We set up an assay for High Throughput Screening based on the HTRF® technology by Cisbio International. 77,000 small-molecule compounds were screened for their ability to inhibit the interaction and several profiles of compounds were identified, pointing to different options for hit to lead optimization. This approach highlights the straightforward way from a complex protein interaction network to the functional validation of a selected interaction as therapeutic target, eventually leading to the identification of small molecules as promising new antiviral drug candidates. (1) S. Emiliani et al. (2005) J. Biol. Chem. 280 : 25517-23

2234/B705

Human Papillomavirus Type 16 E5 Oncogene Affects the Expression of Cell Adhesion and Cell Motility Associated Genes in Epithelial Cells.

E. Auvinen[1,2], N. Kivi[2,1], D. Greco[3], J. Tarkkanen[4], S. Bonsdorff[2,1], P. Auvinen[3]; [1]Department of Virology, Helsinki University Hospital Laboratory, Helsinki, Finland, [2]Department of Virology, Haartman Institute University of Helsinki, Helsinki, Finland, [3]Institute of Biotechnology, University of Helsinki, Helsinki, Finland, [4]Department of Pathology, Helsinki University Hospital Laboratory, Helsinki, Finland

Considerable changes in cellular signaling pathways take place in carcinogenesis. Tumor viruses have mechanisms to modulate crucial cellular signaling pathways for reprogramming host cells and supporting their own life cycle, or for controlling host defense responses. We studied broad-scale effects of the human papillomavirus (HPV) type 16 E5 oncogene in epithelial cells in a genome-wide cDNA microarray screen (Kivi et al., Oncogene 27:2532-41, 2008). We found the expression of altogether 179 genes significantly altered due to the E5 protein in a stable HaCaT human skin keratinocyte cell line transfected with the E5 gene, as compared to control cells transfected with the vector. These included genes involved in cell motility, cell adhesion, cell proliferation, mitogenic signaling, differentiation, and antigen presentation. For example, the expression of matrix metalloproteinases, laminin and lamin A/C was downregulated, and the expression of protein kinase C-delta and p55 regulatory subunit of phosphoinositide-3-kinase proteins were found to be upregulated. We used live-cell microscopy to study whether the changes in cell motility would be observed in vivo. Indeed, cell motility was increased in E5 expressing cells as compared to control cells. Increased motility was shown to be mostly due to enhanced cell movement, not increased cell proliferation. The E5 protein thus seems to affect cellular pathways involved in cell adhesion, cell motility and mitogenic signaling. These alterations may contribute to the inhibition of apoptosis and the establishment of persistent infection and malignant changes in the epithelium. We are now analyzing the effects of the E5 protein on the transcriptional profile of cellular genes as a function of time. We have also performed immunohistochemical stainings in a set of HPV-associated cervical lesions in order to study whether some alterations caused by the E5 gene can be seen at tissue level. Of particular interest are genes involved in epithelial differentiation and the metaplastic process of the human cervix.

2235/B706

Cellular Targets of the Human and Avian Influenza a Virus NS1 Proteins.

M. Thomas[1], P. Massimi[1], G. Matlashewski[2], L. Banks[1]; [1]Tumour Virology Lab, ICGEB, Trieste, Italy, [2]Dept of Microbiology and Immunology, McGill University, Montreal, QC, Canada

Influenza A viruses are the causative agents of seasonal epidemic influenza, which has significant morbidity and causes several thousand deaths each year. It is also the cause of pandemic influenza, which occurs approximately three times per century and can cause millions of deaths and devastating social upheaval. In the 1918 pandemic it has been estimated that up to 100 million people may have died. The 1957 and 1968 pandemics, although severe, resulted in much lower loss of life. Determining the differences between the virus strains causing these pandemics is thus of great importance. In addition to the Haemagglutinin and Neuraminidase proteins that define virus strain, the non-structural protein NS1 has also been the subject of interest. It is a multifunctional virulence factor with several protein-protein interaction domains, including an SH2 domain, an SH3 domain, through which it activates PI3kinase with downstream effects in preventing apoptosis of the infected cell and in evading the interferon response. In addition in >99% of viruses, the NS1 has a class I PDZ-binding motif at the C-terminus, and this has recently been shown to be a virulence determinant. Interestingly, in the majority of human NS1 the consensus motif is RSKV: in avian NS1 it is ESEV. Of the few human strains that have the avian motif, all were from very high mortality outbreaks, including 1918. Previous work has shown us that apparently minor differences in PDZ-binding motifs have major effects on the spectrum of cellular proteins targeted. In this study we analyse the effect of these differences upon the binding of Influenza A virus NS1 protein to a range of cellular proteins involved in polarity and signal transduction.

2236/B707

SumOylation of the Human Cytomegalovirus IE1 Protein Inhibits Its Activity to Interact with STAT2 and Repress Type I Interferon Signaling.

E. Kim, Y. Kim, Y. Huh, J. Ahn; Molecular Cell Biology, Sungkyunkwan University School of Medicine, Suwon, South Korea

In human cytomegalovirus (HCMV)-infected cells, the 72-kDa immediate-early (IE) 1 protein binds to STAT2. This interaction has been suggested to modulate type I interferon signaling during virus infection. IE1 consists of four distinct regions - an amino-terminal region required for nuclear localization, a large central hydrophobic region responsible for PML targeting and transactivation activity, an acidic domain, and a carboxyl-terminal chromatin tethering domain. We found that the 55-amino acid acidic domain of IE1 is required for binding to STAT2. A mutant HCMV encoding the acidic domain-deleted IE1(Δ421-475) was generated. In mutant virus-infected cells, IE1(Δ421-475) failed to bind to STAT2. The growth of mutant virus was only slightly delayed at high multiplicity of infection (MOI), but was severely impaired at low MOI with low level accumulation of viral proteins. When cells were pretreated with interferon β, the mutant virus showed an additional 1,000-fold reduction in viral growth even at high MOI, compared to wild-type. The inhibition of STAT2 loading on the target promoter upon infection was markedly reduced with mutant virus. Therefore, our results provide genetic evidence that IE1 binding to STAT2 requires the acidic domain and promotes viral growth by interfering with interferon signaling. IE1 is covalently modified by SUMO at a lysine residue within the acidic domain. We further investigated the effect of IE1 SUMOylation in the IE1-STAT2 interaction. The SUMO-1 conjugated form of IE1, which was produced in *E.coli*, failed to interact with STAT2 in both in vitro binding assays and virus-infected cells. Furthermore, the C-terminal SUMO-1 fusion of IE1, which was used for mimicry of IE1 SUMOylation, also failed to bind to STAT2 and inhibit the interferon stimulated genes. Therefore, the activity of IE1 to interfere with type I interferon signaling is negatively regulated by a cellular SUMOylation pathway. PIAS1, a SUMO E3 ligase, interacted with IE1 and enhanced SUMOylation of IE1. HCMV IE2, which strongly binds to PIAS proteins, was found to inhibit SUMOylation of IE1 in vitro, suggesting that HCMV may have a mechanism to reduce SUMOylation of IE1.

2237/B708

Changes in Borna Disease Virus Genome with Adaptation to Host.

Y. Nishino[1], S. Okayama[1], N. Miura[1], M. Murakami[2], K. M. Carbone[3], M. Funaba[4]; [1]Laboratory of Veterinary Immunology, Azabu University School of Veterinary Medicine, Sagamihara, Japan, [2]Laboratory of Molecular Biology, Azabu University School of Veterinary Medicine, Sagamihara, Japan, [3]Laboratory of Pediatric and Respiratory Viral Diseases, Center for Biologics Evaluation and Research, U.S. Food and Drug Administration, Bethesda, MD, [4]Laboratory of Nutritional Science, Kyoto University Graduate School of Agriculture, Kyoto, Japan

Borna disease virus (BDV), a nonsegmented negative-strand RNA virus belonging to *Bornaviridae* in the order *Mononegavirales*, is causative to Borna disease exhibiting severe neurological disorder. We have previously reported that CRNP5 variant of BDV had stronger neuropathogenesis in newborn Lewis rats, as compared with CRP3 variant in which only 4 nucleotides in whole genome were different. The CRP3 variant is produced by 3 passages in newborn rat brains of horse-originated BDV, whereas the CRNP5 variant is 5 passages in adult mouse brains after 2 passages in newborn rat brains of the BDV. The present study examined effects of the passage number and host species on the 4 nucleotides differing between CRP3 and CRNP5 and on their pathogenesis. Two nucleotides (nt 3608 and nt 3673) encoding membrane glycoprotein were unaffected during 7 passages in rats, whereas thymidylic acid corresponding to the nucleotides were replaced by cytidylic acid during 3 and 5 passages in mice. Adenylic acid at nt 7936 encoding L polymerase was replaced by guanylic acid in a small population (around 10%) of all BDV variants passaged in rats, whereas 3 passages in mice completely caused replacement to guanylic acid. By contrast, adenylic acid at nt 8742 encoding L polymerase was gradually replaced by guanylic acid during passages in rats, but no replacement occurred during passages in mice. Infection of the BDV variants in newborn Lewis rats revealed that only CRNP5 had severe neurological disease as all rats died within 3 weeks after infection. These results indicate that differences of 4 nucleotides between the BDV variants differently occur during passage of BDV depending on the number and host species, and that the replacement of nucleotides encoding membrane glycoprotein and nt 7936 of L polymerase may be involved in onset of severe neuropathogenesis of CRNP5.

2238/B709

Hantaviruses and TNF-A Act Synergistically to Induce ERK1/2 Inactivation in Vero E6 Cells.

T. Strandin, J. Hepojoki, H. Wang, A. Vaheri, H. Lankinen; Haartman Institute, University of Helsinki, Helsinki, Finland

We have previously reported that the apathogenic Tula hantavirus induces apoptosis in Vero E6 epithelial cells. To assess the molecular mechanisms behind the induced apoptosis we studied the effects of hantavirus infection on cellular signaling pathways which promote cell survival. We previously also observed that the Tula virus-induced cell death process is augmented by external TNF-α. Since TNF-α is

involved in the pathogenesis of hantavirus-caused hemorrhagic fever with renal syndrome (HFRS) we investigated its effects on HFRS-causing hantavirus-infected cells. We observed a direct virus-mediated down-regulation of external signal-regulated kinases 1 and 2 (ERK1/2) survival pathway activity, which was dramatically enhanced by TNF-α. The fold of ERK1/2 inhibition correlated with viral replication efficiencies, which varied drastically between the hantaviruses studied. We demonstrate that in the presence of a cytokine TNF-α, which is increased in HFRS patients, hantaviruses are capable of inactivating proteins that promote cell survival (ERK1/2). These results imply that hantavirus-infected epithelial cell barrier functions might be compromised in diseased individuals and could at least partially explain the mechanisms of renal dysfunction and the resulting proteinuria seen in HFRS patients.

2239/B710

Stable Growth of Hepatitis C Virus in Cell Culture: a Novel Model for Study of Host-Virus Interaction.

P. Ke, S. Chen; Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan

Hepatitis C virus (HCV) is an important human pathogen affecting estimated 3% of the world population. HCV infection causes chronic liver disease, leading to a global health problem. The virological and cellular consequences of persistent HCV infection have been elusive due to the lack of cell-culture system until the recent advance in development of infectious clone. In this study, we report the establishment and characteristics of constitutive-expression of HCV in vitro. The stable expression and production of HCV was achieved by transfection of HCV genotype 2a (JFH1 clone) genome RNAs containing the blasticidin-resistance gene (HCV-Bla) into human hepatoma (Huh7) cells, which was followed by selection with blasticidin. HCV RNA was expressed and replicated efficiently in blasticidin-resistant clones, as shown by the presence of high levels of HCV proteins as well as RNAs. And these stable clones produced HCV-Bla virions with an infectivity up to 103 FFU/ml. Subsequent infection of naïve Huh7 cells with HCV-Bla virus resulted in stable expression of viral proteins and RNAs. Additionally, HCV replication in HCV-Bla transfected cells was specifically suppressed by alpha interferon. Interestingly, we found that that long-term culture of HCV-Bla in stable clones impairs the amplification of infectious titers. Further investigation revealed that CD81 expression was down-regulated in the HCV-Bla stable cells, suggesting the feature of virus-host cell co-evolution. Collectively, these results demonstrate the establishment of a stable HCV culture system that robustly produces infectious virus, which will allow the study of each aspect of the entire HCV life cycle as well as host-virus interaction.

2240/B711

Epithelia Lacking Clathrin Adaptor AP1B Are More Susceptible to Adenovirus Infection.

F. Diaz[1], D. Gravotta[1], A. Deora[1], R. Schreiner[1], J. Schoggins[2], E. Falck-Pedersen[2], E. Rodriguez-Boulan[1]; [1]Ophthalmology, Weill Cornell Medical College, New York, NY, [2]Microbiology, Weill Cornell Medical College, New York, NY

Adenoviruses require the Coxsackie-adenovirus receptor (CAR) and an integrin co-receptor to infect epithelial cells. CAR is a normal component of tight junctions and integrins are usually found at the basolateral surface; hence, epithelial cells are usually highly resistant to infection by adenoviruses from the apical surface, the epithelial domain accessible to viruses invading the organism by digestive or respiratory routes. This study was prompted by our initial observation that different epithelia vary widely in their susceptibility to adenovirus infection. Screening of these epithelia revealed that those highly susceptible to Adv infection, e.g., retinal pigment epithelium, lack AP1B, a clathrin adaptor that resides at Recycling Endosomes. Additional experiments showed that knockdown of AP1B increases the susceptibility of MDCK cells to adenovirus infection ~15 fold. Lack of AP1B promoted apical expression of CAR, due to interference with its recycling (but not with its biosynthetic delivery) to the basolateral membrane, which is mediated by a tyrosine motif of the type recognized by AP1B. Our results provide novel insight on the cellular machinery responsible for normal sorting of a viral receptor and illustrate how variations in such machinery result in changes in tissue-specific infection. Supported by NIH, Research to Prevent Blindness Foundation and the Dyson Foundation.

2241/B712

Virus Infection-Induced Alterations in Nuclear Dynamics.

T. O. Ihalainen[1], E. Niskanen[2], O. Paloheimo[3], J. Laakkonen[4], T. Turpeinen[5], J. Timonen[6], M. Vihinen-Ranta[7]; [1]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland, [2]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland, [3]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland, [4]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland, [5]Department of Physics, University of Jyväskylä, Jyväskylä, Finland, [6]Department of Physics, University of Jyväskylä, Jyväskylä, Finland, [7]Department of Biological and Environmental Science, University of Jyväskylä, Jyväskylä, Finland

Canine parvovirus replication proceeds in parvovirus-associated replication (APAR) bodies formed in a dynamic process starting from the appearance of nonstructural protein (NS1) containing foci and proceeding to emergence of compartments extending throughout the nucleus. Confocal microscopy imaging of infected Norden Laboratory Feline Kidney cells combined with colocalization analysis showed time-dependent increase in NS1 interaction with nuclear component, speckle marker (PABP2), proliferating cell nuclear antigen (PCNA) and transcription factor 2B (TF2B). Fluorescence recovery after photobleaching (FRAP) demonstrated that recovery kinetics of PABP2 and TF2B were significantly slower in infected cells than in noninfected cells thus suggesting for virus-induced alterations in binding and diffusion dynamics. NS1 fused with enhanced yellow fluorescence protein (NS1-EYFP) provided a marker in live cells for dynamics of NS1. FRAP suggested that the NS1 protein is not freely diffusing but undergoes transient interactions with nuclear components both in the presence or absence of infection. However, in infected cells mobility of NS1 is more restrained than in noninfected cells thus reflecting increased interaction or transient complex formation with other viral components. Further analysis of FRAP data by mathematical modelling suggested that binding sites of NS1 in APAR bodies are mobile. Moreover, live cell analysis of infected cells expressing the core histone H2B protein fused to ECFP revealed that formation of APAR bodies was accompanied by time-dependent remodelling and margination of the host cell chromatin. Taken together, these results highlight the dynamic nature of NS1-induced interactions and nuclear reorganization.

2242/B713

The Drosophila RNAi Entry Pathway and Its Role in Antiviral Immunity.

A. N. Gillis[1], M. Saleh[2], R. Andino[1]; [1]Cell Biology, UC San Francisco, San Francisco, CA, [2]Virology and RNAi, Institute Pasteur, Paris, France

RNA interference (RNAi) is a widely conserved nucleic acid-based gene regulatory mechanism. Recent studies have shown that RNAi is an essential component of the antiviral immune response in plants, C. elegans and Drosophila melanogaster. We have previously performed a genome-wide screen in Drosophila S2 cells that demonstrated that cells readily take up long double stranded RNA (dsRNA) from the extracellular environment by receptor-mediated endocytosis and use it for RNAi-based gene silencing. To better understand the RNAi entry pathway and determine its role for antiviral defense, we are now characterizing the candidate genes using high-resolution microscopy and biochemical techniques. In order to determine if RNAi entry is required for antiviral immunity in vivo, we are studying flies carrying mutations in the candidate genes for susceptibility to viral infection. We will present the results obtained using electron microscopy of dsRNA and by tracking the subcellular localization of dsRNA after internalization.

2243/B714

Real Time In Vivo Visualization of Bacteriophage Lambda DNA Ejection.

D. Wu, H. Garcia, A. Tadmor, H. Lee, R. Phillips; California Institute of Technology, Pasadena, CA

After bacteriophage lambda binds to *E. coli*, it injects its DNA and either becomes a prophage or starts synthesizing phage precursors eventually leading to cell lysis. The whole (latter) process takes on the order of 30 minutes. We focus here on the initial events in the infection process during which the viral genome is translocated into the host bacterium. While DNA ejection has been visualized *in vitro*, it has yet to be seen *In Vivo*. We report here on visualizing the ejection of phage DNA at the single molecule level into its host in real time. Using DNA free minicells loaded with DNA-staining dye, we are able to visualize the rate of injection. The events are also visualized by engineering phage with binding sites for fluorescent DNA binding proteins. We additionally use optical tweezers to control the initial time of phage-bacteria binding to measure the waiting time between phage binding and DNA ejection. Our preliminary results indicate that the *In Vivo* ejection process proceeds at a much slower rate than *in vitro*. In summary, by elucidating the rate of DNA ejection, we can better understand the requirements for lambda phage infection beyond the necessary condition of attachment to its receptor, as well as refine our polymer physics models of DNA translocation.

2244/B715

Effects of Adenovirus Super-Infection on Transcription and Translation from a Gene under the MLP and Tripartite Leader Sequence.

M. El-Mogy[2], Y. Haj-Ahmad[2,1]; [1]Norgen Biotek Corp., Thorold, ON, Canada, [2]Brock University, St. Catharines, ON, Canada

Adenoviruses are commonly used as a tool for the study and understanding of gene expression, oncogenic transformation, DNA replication, gene delivery and other molecular biological phenomenon. Upon infection of permissive cells and during the late phase of the viral life cycle the adenovirus major late promoter (MLP) is the predominant promoter, and together with the tripartite leader sequence (TPL) is responsible for the abundant late viral protein production. In this study, we investigated the effect of the incomplete and complete TPL sequence on mRNA transcription and translation under the control of the adenoviral wild type super-infection. A series of plasmids containing the MLP, with partial and complete TPL exons (exon 1, exon 1 & 2 and exon 1, 2 & 3) were used and gene expression was evaluated in Chinese hamster ovary (CHO) cells using the green fluorescence protein (GFP) as a reporter gene. After plasmid transfection and adenoviral wild type super-infection, GFP mRNA transcription levels were measured over time via RT-qPCR. Translation levels were determined by direct ELISA using anti-GFP antibodies. Following transfection, no significant changes in mRNA transcription efficiency were obtained with the incorporation of the different TPL exons. On the other hand, wild type super-infection showed a minimum effect on transcription efficiency induction from cassettes without TPL or with only the first TPL exon. Induction of transcription efficiency was observed when using TPL exons 1+2 or the complete TPL (exons 1+2+3). Similarly, protein translational levels showed the same trend, with maximum levels when the complete TPL form was used. These results emphasize the effect of wild type adenovirus trans-activation on MLP and TPL activity and the importance of the complete TPL structure on mRNA stability and function.

2245/B716

PVY Resistant Ry Gene Differences between Potato Cultivars.

J. Park, S. Lee, Y. Yang, H. Cheong; Chosun University, Gwangju, South Korea

Potato Virus Y (PVY) is the type member of the genus *potyvirus* in the family. PVY infected potato shows several symptoms like mosaic, yellowing, necrosis, mottle, vein clearing, vein bending and misshapen. Also PVY infected potato loss crop yield 20 to 50 percent. Furthermore PVY has decrease crop yield 80 percent when PVY co-infected with other potato virus such as PVX. We had identified DNA sequence of the *cv. Golden Valley* in our previous result and amplified *Ry* gene from *cv. Winter* and *Gogu Valley*. To confirm the DNA sequence, each fragment of *Ry* gene was inserted in pGEM-T easy vector. The DNA sequence of *Winter Valley* and *Gogu Valley* was analogous to that of *Golden Valley Ry* gene and *Solanum tuberosum subsp. andigena Ry*-1 gene.

Symposium VII: Gene Regulation and Non-Coding RNAs (2246 – 2248)

2246

Varied Functions of Small, Non-Coding RNAs in Bacteria.

G. Storz, S. Durand, F. Fontaine, E. M. Fozo, J. A. Opdyke, L. S. Waters, A. Zhang; Cell Biology and Metabolism Program, NICHD/NIH, Bethesda, MD

In recent years, a combination of computational and molecular approaches has led to the identification of >80 small, noncoding RNAs in E. coli. Increasing numbers of small RNAs are also being discovered in other bacteria. The small RNAs that have been characterized act by two general mechanisms. A subset of the small RNAs bind proteins and modify their activities. The others act through basepairing with mRNAs. In some cases the region of complementarity is extensive while in other cases only short regions of complementarity are required for regulation. Basepairing can lead to increased or decreased message stability or can block or facilitate translation. The mechanisms of small RNA action and their physiological roles will be discussed. Supported by the Intramural Program of the NICHD.

2247

Nuclear and Chromatin Dynamics during X-Chromosome Inactivation.

E. Heard; Nuclear Dynamics and Genome Plasticity, Institut Curie/UMR 218, CNRS, Paris cedex 05, France

X inactivation is a dramatic example of monoallelic gene regulation on a chromosomes-wide scale. We are interested in the basis of the differential treatment of the two X chromosomes within the same nucleus during the initiation of X inactivation, as well as the mechanisms that subsequently provide the cellular memory of the inactive state. X inactivation occurs during early development and in mice is a highly dynamic process. In addition to epigenetic modifications, nuclear localization and compartmentalization are implicated both in the initiation and in the maintenance of X inactivation. We recently demonstrated that the locus that controls the initiation of X inactivation (the Xic) is involved in dynamic trans-interactions that may enable a cell to sense the number of X chromosomes that are present and trigger the X-inactivation process. Different regions of the Xic are involved in this pairing process. In particular, we have identified a new Xic region that drives initial co-localisation of the Xics and may participate in regulating X inactivation. We have also recently shown that the non-coding Xist transcript produced by the Xic, that is responsible for initiating X inactivation, may function by spatially segregating the X chromosome it associates with. Xist RNA forms a transcriptionally silent, repeat-rich nuclear compartment very early on in the X-inactivation process and genes then become recruited into this compartment when they are silenced. The role of repeats in X inactivation is thus being investigated. Our progress in understanding the spatial dynamics of the Xic, as well as the chromatin changes and nuclear reorganization of the X chromosome induced by the Xist transcript, will be presented.

2248

Conserved Roles of Small RNAs in Genome Defense.

G. Hannon; Cold Spring Harbor Laboratory/HHMI, Cold Spring Harbor, NY

The diversity of functions for small RNAs has been expanding consistently since their discovery some 7 years ago. Recently, we have focused on the roles of small RNAs in the germline of both mammals and invertebrates. Through studies of a clade of Argonaute proteins, which shows largely gonad-restricted expression patterns, we identified a class of small RNAs with conserved roles in transposon suppression. These piRNAs form the basis of an elegant adaptive immune system that silences transposons at post-transcriptional level, and also either directly or indirectly at the transcriptional level in some cases. More recently, studies in the female germline of mammals have revealed yet another class of small RNAs, which appear to be endogenously generated siRNAs. These small RNAs, which are often generated either from pseudogenes or gene-pseudogene interactions have demonstrable roles in regulating gene expression. Thus, endogenous siRNA systems that exist in other animals appear also to extend to mammals. Moreover, the existence of small RNAs, with near perfect complementarities to their regulatory targets, might provide at least one explanation for the conservation of Argonaute-mediated catalysis in mammals.

Education Initiative Forum III: The Young Scientist Program at Washington University in St. Louis (2249)

2249

The Young Scientist Program at Washington University in St. Louis.

A. L. Buchwalter[1], M. R. Beck[4], B. L. Moss[3], J. Mosher[5], T. A. Woolsey[2]; [1]Cell Biology and Physiology, Washington University in St. Louis, St. Louis, MO, [2]Neurological Surgery, Washington University in St. Louis, St. Louis, MO, [3]Mallinckrodt Institute of Radiology, Washington University in St Louis, St Louis, MO, [4]Biochemistry and Biophysics, University of North Carolina- Chapel Hill, Chapel Hill, NC, [5]Young Scientist Program, Washington University in St. Louis, St. Louis, MO

The Young Scientist Program (YSP) is a science education outreach program run by graduate and medical students at Washington University in St. Louis, Missouri. YSP aims to improve science education in local middle and high schools and attract young students to scientific careers. Student volunteers participate for reasons including a desire to participate in community outreach, to gain teaching experience, and to increase the participation of underrepresented groups in science. YSP volunteers have developed several programs to supplement the educational experience of students and their teachers. *Teaching Teams*: YSP volunteers develop and lead inquiry-driven science modules in local middle and high school science classes and after-school programs. Teaching Teams are active in the areas of Anatomy, Chemistry, Genetics & Genomics, Ecology & Evolution, Forensics, Microbiology, Neuroscience, and Physics. These science modules are modified in collaboration with teachers to integrate classroom curricula and state standards. Teaching teams have interacted directly with more than 5000 students since 1993. *Summer Focus*: Local high school students are encouraged to apply to YSP's Summer Focus program, which places the

student in a summer laboratory internship at the university. Each Summer Focus Scholar is mentored by a volunteer graduate student or postdoctoral fellow, completes an independent research project, and writes a formal research paper during the eight-week internship. More than 165 local students have participated in Summer Focus. *Teacher and Researcher Partnership*: YSP initiated this program in 2001 to provide summer research and curriculum enrichment experiences for public school teachers. Teacher participants work with mentors on an individual research project and on development of science activities for their own classrooms. *Benefits and Outcomes*: YSP asks its participants to evaluate their experience in order to continuously assess the effectiveness of YSP's programs. Volunteers report gaining a variety of skills that are not formally taught during graduate and post-graduate training. Student and teacher participants report an increased interest in and understanding of scientific concepts.

Symposium VIII: Models for Stem Cell Biology (2250 – 2252)

2250

C. Elegans Germline Stem Cells and Their Regulation.

J. Kimble[1,2]; [1]Howard Hughes Medical Institute, Madison, WI, [2]Department of Biochemistry, University of Wisconsin-Madison, Madison, WI

Stem cells maintain themselves in a process called self-renewal, and they also generate differentiated cells to replenish tissues. As a result, stem cells hold great promise for the clinic — promise to repair tissues that are worn out, damaged or diseased and promise to make new tissues and perhaps even new organs for replacement. On the flip side, stem cells wreak havoc for the organism if they are released from normal constraints. Therefore, an indepth understanding of stem cell regulation has significant clinical implications. One major stem cell control that operates within the organism is the stem cell niche, a 'microenvironment' responsible for stem cell maintenance. My laboratory investigates the development and function of a stem cell niche as well as how stem cells respond to signaling from the niche. To this end, we employ the experimentally tractable nematode *Caenorhabditis elegans*. In this small worm, a single mesodermal cell, the distal tip cell (DTC), provides the stem cell niche for germline stem cells. We have found that conserved signal transduction pathways are critical for DTC specification during development and for niche signaling to stem cells both during development and in adults. We have also delineated a regulatory network that responds to niche signaling and thereby controls the balance between stem cell maintenance and differentiation. This network relies heavily on RNA regulation, a theme that pervades germ cell biology. Of particular importance are PUF (for Pumilio and FBF) RNA-binding proteins, which control stem cell maintenance in many animals, perhaps including mammals. Indeed, many stem cell regulators found in the *C. elegans* model system have been implicated in stem cell control in mammalian tissues. The DTC regulation of germline stem cells therefore appears to be a superb model for identification and analysis of fundamental stem cell controls.

2251

The Dynamics of Neural Stem and Progenitor Cells in Cortical Development.

A. Kriegstein; Institute for Regeneration Medicine, University of California, San Francisco, San Francisco, CA

Recent work has identified the neural stem cells in the embryonic and adult brain and is unraveling the mechanisms by which new nerve cells are created and delivered to their correct locations. In addition, insights gained from studies of the developing brain are helping to guide efforts to create unlimited numbers of replacement nerve cells from stem cell lines. Radial glial cells, present only in the embryonic and fetal brain and long thought to simply guide embryonic nerve cells during migration, have now been identified as neuronal stem cells in the developing brain. Neurogenic radial glia are highly interactive, communicating with each other directly through gap junction channels and responding to their local environment through specific neurotransmitter receptors. Radial glia divide asymmetrically to self-renew and produce nerve cells that often climb along their parent radial glial cells to reach the developing cerebral cortex. Radial glia also produce neurons through intermediate progenitor cells that are the product of radial glial cell divisions and undergo symmetric modes of cell division in the subventricular zone of the embryonic cortex. These symmetric divisions increase the numbers of cells within the same layer that are of a given type. This two-step process of neurogenesis suggests new mechanisms for the generation of cell diversity and cell number in the developing cortex. Additionally, production of neurons through intermediate progenitor cells suggests strategies for generating specific neuron types for cell replacement therapies.

2252

The Epigenetic Role of Piwis and Pirnas in Stem Cell Self-Renewal.

H. Lin; Yale Stem Cell Center, Yale University, New Haven, CT

The hallmark of stem cells is their ability to self-renew meanwhile capable of producing numerous differentiated daughter cells. Previously, we discovered the ARGONUATE/PIWI protein family that is essential for stem cell self-renewal in diverse organisms. This protein family can be divided into ARGONUATE and PIWI subfamilies. Recently, we and others demonstrated that the PIWI subfamily proteins interact with a novel class of non-coding small RNAs that we named PIWI-interacting RNAs (piRNAs). piRNAs are 26~30 nucleotides in length, and are abundantly expressed during spermatogenesis. Individual piRNAs frequently correspond to intergenic and repetitive sequences. In addition, a small number of piRNAs correspond to exonic and intronic sequences. This broad genomic distribution of piRNAs implicates their potential involvement in diverse mechanisms of gene regulation during spermatogenesis. I will present our recent progress on how PIWI subfamily proteins and piRNAs are involved in epigenetic regulation that controls the self-renewing fate of germline stem cells.

Minisymposium 25: Actin-based Motors (2253 – 2258)

2253

The In Vivo Motion, Processivity, and Stepping of Quantum Dot-Labeled Single Myosin Va Molecules.

S. Nelson, M. Ali, K. Trybus, D. M. Warshaw; Molecular Physiology & Biophysics, University of Vermont, Burlington, VT

Myosin Va (myoVa) is an intracellular cargo transporter. Based on in vitro experiments, a single myoVa molecule could perform this function as it can move processively along actin tracks in a "hand-over-hand" fashion. However, this has yet to be demonstrated in vivo. Numerous intracellular challenges and obstacles to myoVa processivity are presented by the dense cytoskeletal network, actin-binding proteins, and other motors that share cargo transport duties with myoVa, but generate either assistive or resistive loads. To study the in vivo motion and processivity of myoVa, we introduced quantum dot (Qdot) labeled, exogenously expressed myoVa HMM into COS-7 cells by pinocytosis, and observed individual myoVa molecules by TIRF microscopy. Only Qdots within the cell that are conjugated to a functional myoVa molecule are observed in the TIRF field. Although the majority of Qdot-myoVa movement appears random, mean squared displacement analysis and subsequent visual inspection reveals the presence of periods (0.5-1.0s) of linear, unidirectional motion, resulting from an active process that is ATP-sensitive. With high time (16ms) and spatial (15nm) resolution imaging, the movement was characterized by a mean velocity of 600nm/s, regardless of the number of myoVa molecules attached to the Qdot and similar to what is measured with this construct in vitro. Individual Qdot-myoVa molecules take sequential 75nm steps at a rate of 16s-1. The 75nm step is expected for a myoVa molecule that has a Qdot on only one of its heads, while the stepping rate is similar to the ADP-release rate, which is the rate limiting step for processive movement. Although the in vivo myoVa trajectories appear random at more macroscopic scales, this may be attributed to a processive myoVa motor undergoing a random walk through the dense cortical actin network. Using this approach, we can begin to define how myoVa is targeted to its cargo and how multiple myoVa motors coordinate their activity to bring about efficient cargo transport.

2254

Single-Molecule Imaging of Myosin-X in Living Cells Reveals a Novel Form of Motility.

M. L. Kerber[1], D. Jacobs[1], L. Campagnola[1], T. Yin[1], O. Quintero[1,2], R. Cheney[1]; [1]Cell and Molecular Physiology, UNC - Chapel Hill, Chapel Hill, NC, [2]Biological Sciences, Mt. Holyoke College, South Hadley, MA

Myosin-X (Myo10) is an actin-based molecular motor hypothesized to use its motor activity to move forward along actin filaments to the tips of filopodia. Here we use TIRF microscopy to detect and track the movements of motor molecules with single molecule sensitivity in living cells. TIRF reveals a novel form of motility in which extremely faint particles of GFP-Myo10 move in a rapid and directed fashion towards the filopodial tip. These particles move forward at 578 +/-174 nm/s at 25 C and at 840 +/- 210 nm/s at 37 C. These particles thus move forward 5-10 fold faster than the large puncta of Myo10 detected previously with widefield microscopy. TIRF also allows detection of faint particles of GFP-Myo10 that move rearward at the actin retrograde flow rate of 10-20 nm/s. Based on their diffraction-limited size, intensity, and stepwise bleaching, the faint particles appear to correspond to individual Myo10 molecules. Particle movements are not observed for headless Myo10, demonstrating that the motor domain is necessary for particle movement. The Myo10 tail is not necessary since an HMM-like Myo10 construct exhibits forward and rearward movements similar to those of full length Myo10. The particle movements are blocked by 1 uM latrunculin B, but not by 5 uM nocodazole, indicating that this process is dependent on actin, but not microtubules. Interestingly, faint particles of GFP-Myo5a exhibit similar forward movements in filopodia, but these occur at only 251 +/- 121 nm/s. Faint particles of Myo5a also move rearward at 10-20 nm/s, demonstrating that Myo5a is capable of coupling to actin retrograde flow. GFP-Myo1a did not exhibit clear forward or rearward movement, indicating that not all myosins are capable of intrafilopodial motility. These data highlight the usefulness of filopodia for single molecule studies in living cells and suggest the existence of a novel system of forward transport within filopodia. (The first two authors contributed equally to this work. Supported by NIH/NIDCD grant DC03299 to REC and NIH/NHLBI HL080166)

2255

Two Modes of Cargo Attachment to Myosin V Motors.

Y. Jin[1], M. J. Brunner[1], Y. Peng[1], P. T. Eves[1], Z. Lipatova[2], A. A. Tokarev[2], N. Segev[2], A. Fagarasanu[3], R. Rachubinski[3], L. S. Weisman[1]; [1]Cell and Developmental Biology, Life Sciences Institute, The University of Michigan, Ann Arbor, MI, [2]Department of Biological Sciences, Laboratory for Molecular Biology, The University of Illinois at Chicago, Chicago, IL, [3]Department of Cell Biology, University of Alberta, Medical Sciences Building 5-14, Edmonton, AB, Canada

Myosin V motors move cargoes along actin filaments. Within some cell-types, a single isoform of myosin V moves multiple cargoes to distinct places at different times. The distribution of cargoes to their proper location is achieved in part through regulation of myosin V attachment to organelle specific receptors. Based on the high-resolution structure of the myosin V cargo-binding domain and site-directed mutagenesis, we mapped surface residues required for interaction of myosin V to specific cargoes. Here, we report two conserved sites on the cargo-binding domain of myosin V that regulate cargo attachment in two different ways. The sites are located at opposite ends of the cargo-binding domain and are offset by 180°. One site, utilized by secretory vesicles, binds directly to a Rab GTPase; this binding requires that the Rab is bound to GTP. The second site, utilized by the yeast vacuole/lysosome, is independent of Rab GTPases and instead binds directly to an adaptor protein. This interaction is regulated through direct phosphorylation of the adaptor by the cyclin-dependent kinase Cdk1. Notably, each of the two sites on myosin V is involved with binding more than one type of cargo. We also mapped the binding sites of additional organelle specific receptors. Notably, each binds to surface residues on myosin V that are shared with either the vacuole or secretory vesicle binding site. These findings suggest that a subset of cargoes compete with each other for access to myosin V. In addition, the direct involvement of a Rab GTPase in the attachment of some cargoes, suggests that in those cases membrane movement and subsequent fusion are coordinated.

2256

Drosophila Myosin 7a Is Regulated by Intramolecular Folding.

Y. Yang[1], T. Baboolal[2], V. B. Siththanandan[1], P. J. Knight[2], M. Peckham[2], J. R. Sellers[1]; [1]Laboratory of Molecular Physiology, National Heart, Lung and Blood Institute, Bethesda, MD, [2]School of Biomedical Sciences, University of Leeds, Leeds, United Kingdom

Full length Drosophila myosin 7a (myo7a-FL) has a complex tail structure containing a short putative coiled-coil followed by a MyTH4-FERM domain followed by an SH3 domain and then a second MYTH4-FERM domain. Myo7a-FL expressed in Sf9 cells is monomeric despite the presence of the predicted coiled-coil. We have previously shown that a Subfragment-1 (S1) from this myosin has a Vmax of 1 per sec and a Km of 1μM. Myo7a-FL has a Vmax similar to that of S1, but its Km is 40μM. Thus, at low actin concentrations (5μM), the MgATPase activity of S1 is fully activated, whereas that of myo7a-FL is still very low, indicating regulation of the activity. Furthermore, myo7a-FL does not move actin filaments in vitro at 50mM KCl. Negatively-stained processed images of myo7a-FL in the presence of ATP show that the tail is tightly folded against the motor domain forming a compact structure which obscures the normally easily recognized features of the motor domain. Myo7a-FL unfolds at either high ionic strength or in the absence of ATP, revealing a clearly recognizable motor domain, the lever arm and some features of the tail region. C-terminal truncations were made to determine which portions of the multi-domained tail are necessary for the regulation. Removal of the last 99 amino acids which are highly conserved in all myo7 molecules, and form a subdomain (termed MyTH7) of the FERM domain, is sufficient to prevent folding of the molecule in the presence of ATP. The Km of this mutant is similar to that of the S1 fragment. Other, larger C-terminal truncations have similar effects. Point mutations of a two conserved charged residues in this region, R2140 and K2143, abolish the folding and result in activation of the MgATPase by low concentrations of actin. Interestingly, a construct consisting of the second FERM domain binds actin in an ATP-insensitive manner with a Kd of around 30μM which is similar to the KATPase value for the full length molecule. We propose a model where, at low actin concentration myo7a-FL is folded and inactive, but at high actin concentrations such exists in actin bundles, myo7a-FL binds first via its tail binding site which then frees the motor domain to functionally interact with actin.

2257

Myosin-1a Regulates the Release of Vesicles Enriched in Host Defense Machinery into the Intestinal Lumen.

R. McConnell[1], J. N. Higginbotham[2], R. Coffey[1,2], M. Tyska[1]; [1]Cell and Developmental Biology, Vanderbilt University Medical Center, Nashville, TN, [2]Medicine, Vanderbilt University Medical Center, Nashville, TN

The enterocyte brush border is composed of a tightly packed array of microvilli and has classically been viewed as a passive cytoskeletal scaffold that serves to increase apical membrane surface area of the intestinal epithelium. However, recent work with isolated brush borders indicates that the actin-based motor protein, myosin-1a, powers the release of apical membrane vesicles from the tips of microvilli, a process we now refer to as brush border membrane shedding. The goal of this study was to investigate the function of this novel aspect of enterocyte biology and the contribution of myosin-1a to this process in vivo. Using the luminal contents of the small intestine as starting material, we employed biochemical fractionation and fluorescence-activated vesicle sorting to purify "luminal vesicles" released from the enterocyte during brush border membrane shedding. These vesicles contain a number of catalytically active enzymes and other proteins ordinarily associated with the brush border. In mice lacking myosin-1a, luminal vesicles are significantly fewer in number, larger in diameter, and have altered protein composition. Interestingly, one of the most highly enriched luminal vesicle components is intestinal alkaline phosphatase, a brush border enzyme recently implicated in mucosal defense against toxic bacterial products. Consistent with a role in host defense, these vesicles physically interact with luminal bacteria, dephosphorylate (i.e. detoxify) purified bacterial lipopolysaccharide, and protect cultured epithelial cells from the pro-inflammatory signaling normally induced by this microbial product. These data indicate that myosin-1a regulates the formation of luminal vesicles and further suggests that brush border membrane shedding is a novel and previously uncharacterized mechanism for distributing host defense machinery into the intestinal lumen.

2258

Mechanosensing through Cooperative Interactions between the Motor Myosin-II and Actin Crosslinker Cortexillin-I.

Y. Ren[1], J. Effler[1], R. Firtel[5], P. Iglesias[4], D. Robinson[1,2,3]; [1]Cell Biology, Johns Hopkins University School of Medicine, Baltimore, MD, [2]Pharmacology and Molecular Sciences, Johns Hopkins School of Medicine, Baltimore, MD, [3]Chemical and Biomolecular Engineering, Johns Hopkins University, Baltimore, MD, [4]Electrical and Computer Engineering, Johns Hopkins University, Baltimore, MD, [5]Section of Cell and Developmental Biology, UCSD, La Jolla, CA

Mechanosensing is emerging as a critical principle that governs how processes from the molecular to organismal levels function. We recently discovered a mechanosensory shape control pathway that senses and corrects cell shape during cell division in the protozoan Dictyostelium. Here, we have identified a critical relationship between the actin crosslinker cortexillin-I and myosin-II, which form the basis of this mechanosensory module. Both myosin-II and cortexillin-I require each other in order to be recruited to cortical sites of increased mechanical strain. Though many biochemical functions of myosin-II are not essential for cleavage furrow ingression, all functions (heavy chain thick filament regulation, light chain phosphorylation, and wild type mechanochemistry and neck lever) are required for full mechanosensing capability. Similarly, minimal domains of cortexillin-I are insufficient to support mechanosensing ability. In particular, deletion of the amino-terminal calponin homology domain of cortexillin-I leads to a protein that is fully functional for cleavage furrow ingression but is partially compromised for mechanosensing ability. The sum of the data suggests a model in which myosin-II and cortexillin-I communicate through the actin filament to direct their co-localization in response to mechanical strain.

Minisymposium 26: Cell-Cell Communication (2259 – 2264)

2259

Structure of a Complete Integrin Ectodomain in a Physiologic Resting State and Activation and Deactivation by Applied Forces.

T. Springer[1], Z. Zhu[1], B. Luo[2], T. Xiao[3], C. Zhang[1], N. Nishida[4]; [1]Immune Disease Institute, Department of Pathology, Harvard University School of Medicine, Boston, MA, [2]Department of Biological Sciences, Louisiana State University, Baton Rouge, LA, [3]Laboratory of Immunology, Structural Immunobiology Section, National Institute of Allergy and Infectious Diseases, Bethesda, MD, [4]Graduate School of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Japan

An atomic structure of a complete integrin ectodomain is important to understand conformational change, and whether there is a structural basis for linking cystoskeleton attachment to high affinity for ligand. A crystal structure of integrin αIIbβ3 ectodomain in a bent, low-affinity conformation reveals all of its domains including those in the β-knee. Metal ions required for ligand binding are pre-loaded. Different integrin molecules in crystals and EM reveal breathing that appears on pathway to extension. With the α and β integrin subunits associated proximal to the membrane, tensile force applied to the ligand-receptor complex in the extended conformation stabilizes the closed, low-affinity headpiece conformation. By contrast, an additional lateral force applied to the β subunit to mimic attachment to moving actin filaments stabilizes the open, high-affinity headpiece conformation. Integrin structure and tensile forces thus provide mechanisms for propagating allostery over long distances and coupling the high affinity state to attachment to the cytoskeleton.

2260

Structure and Association of the Integrin αIIb and β3 Transmembrane Segments.

T. Lau[1], M. Ginsberg[2], T. S. Ulmer[1]; [1]Department of Biochemistry & Molecular Biology, University of Southern CA, Los Angeles, CA, [2]Department of Medicine, University of CA, San Diego, San Diego, CA

Integrin cell-adhesion receptors transduce signals bidirectionally across the plasma membrane via the single-pass transmembrane segments of each α and β subunit. Here, we report the high-resolution structure of the individual αIIb and β3 integrin transmembrane segments in phospholipid bicelles and provide information on their heterodimeric association. The monomeric β3 transmembrane segment is found to adopt a 30-residue linear α-helix, encompassing residues Ile693-His772, of which Ile693-Ile721 appear embedded in the hydrophobic bicelle core. This relatively long transmembrane helix implies a pronounced helix tilt within a typical lipid bilayer, which facilitates the snorkeling of Lys716's charged side chain out of the lipid core while simultaneously immersing hydrophobic Leu717-Ile721 in the membrane. The structure of the individual αIIb transmembrane segment reveals a linear 24-residue α-helix (Ile966-Lys989) followed by a backbone reversal that packs Phe992-Phe993 against the transmembrane helix. The length of the αIIb transmembrane helix implies the absence of a significant transmembrane helix tilt in contrast to its partnering β3 subunit. Sequence alignment shows Gly991-Phe993 to be fully conserved among all 18 human integrin α subunits, suggesting that their unusual structural motif is prototypical for integrin α subunits. The Gly991-Phe993 motif disposes α subunits towards a short and straight transmembrane helix, whereas the five mostly hydrophobic residues following the first charged residue, e.g. β3(Lys716), in β subunits biases them toward a long and tilted transmembrane helix. The here reported structures of the αIIb and β3 transmembrane helices define the activated state of integrin receptors and form the structural basis for assessing the extent of structural and topological rearrangements upon αIIb-b3 association, i.e. integrin transmembrane signaling. Data on the structure of the associated αIIb-β3 transmembrane state will be presented.

2261

Quantifying Protein-Mediated Cell Adhesion by Single Cell Force Spectroscopy.

T. Mueller, G. Behme, H. Haschke, R. Owen; JPK Instruments AG, Berlin, Germany

The CellHesion Development Kit has been designed to allow reproducible and quantitative analysis of cell-cell and cell-substrate binding forces. For two examples it will be demonstrated how single force spectroscopy can used to analyse the function of galectins in epithelial cells as well integrin-mediated cell adhesion (CHO) to a collagen I matrix. Using a combination of light microscopy and force spectroscopy, single cells can be selected, attached to a flexible cantilever and subsequently allowed to adhere to a second, specific cell or region of substrate. The instrument allows precise control of the force the cells are subjected to during a user-defined binding period under temperature control. The bound cells can then be separated at a specific speed, to a distance of up to 100 µm. By monitoring the bending of the sensor during the retraction process a force-distance curve can be plotted. From this force-distance curve it is possible to calculate the amount of work and maximal force required to separate single cells from each other or from substrate. Additionally, analysis of sub-features of the force-distance retraction curve allows the determination of the force required for the unbinding of single protein-protein interactions at the cell surface. The batch processing software of force-distance-curves accelerates the data analysis as well their comparability. Step fitting algorithms facilitate the analysis of tether pulling forces and their numbers. The combination of single cell force spectroscopy with phase contrast, DIC and epifluorescent microscopy or confocal imaging enable the selection of specific cells within a culture for binding measurements, but also the monitoring of multiple additional cellular processes that may occur on binding, such as changes to actin structure, calcium flushes, distribution of labelled proteins, or morphological changes.

2262

Cell Communication Controlling Chemotactic Cell Movement during Dictyostelium Development and Chick Gastrulation.

C. Weijer[1], M. Chuai[1], G. Singer[1], X. Yang[2], D. Dormann[3]; [1]School of Life Sciences, Division of Cell and Developmental Biology, Weijer Lab, University of Dundee, Dundee, United Kingdom, [2]Medical College, Jinan University, Guangzhou, China, [3]MRC Clinical Sciences Centre, Imperial College, London, United Kingdom

We investigate the molecular mechanisms by which cells produce and detect chemotactic signals and translate this information in directed coordinated movement up or down chemical gradients in the social amoebae Dictyostelium discoideum, and during gastrulation in the chick embryo. During Dictyostelium development hundreds of thousands of cells aggregate chemotactically to form a fruiting body. Chemotactic cell migration during all stages of development is controlled by propagating waves of the chemo-attractant cAMP. Using a variety of imaging techniques we analyse the spatio-temporal dynamics of cAMP mediated cell-cell signalling and the resulting chemotactic cell movement responses to understand how the interactions beween cell-cell signalling and movement govern the multi-cellular organisation of this organism. Investigation of mutants that change cAMP cell-cell signalling dynamics and the organisation and function of the actin-myosin cytoskeleton affecting chemotactic movement, show how cellular heterogeneity in signalling dynamics and polarised activation of the actin-myosin cytoskeleton drive aggregation, cell sorting, slug formation and migration. Chemotactic cell movement also plays a critical role during gastrulation in the chick embryo. Our experiments show that epiblast cell movement during the formation of the primitive streak as well as the movement of the mesoderm cells after their ingression through the streak is controlled by a combination of attractive and

repulsive guidance cues. We have identified FGF4 and VEGFA as attractive cues for mesoderm cells, while FGF8 and Wnt's act as repulsive signals. We are currently trying to establish how signalling and movement interact.

2263

Ap-1 Clathrin Adaptor Complex Controls the Localization of the Notch-signalling Regulator Sanpodo in Drosophila Sensory-Organ Precursor Cells.

N. Benhra, S. Lallet, R. Le Borgne; Developmental Cell Biology, IGDR-CNRS UMR 6061-Faculté de Médecine, Rennes, France

During metazoan development cell fate diversity is in part generated via asymmetric cell division, in which mother cells divide to produce two daughter cells with distinct developmental potential. In Drosophila, the sensory organ precursor cell called pI divides four times asymmetrically to generate the sensory organ lineage. During pI division, the cell fate determinant Numb segregates asymmetrically at the anterior cortex and is unequally inherited by the anterior daughter cell. Numb is proposed to negatively regulate Notch signalling pathway by promoting the endocytosis of the four-pass transmembrane protein Sanpodo (Spdo). Thus the anterior pI daughter cell adopts the pIIb fate. By contrast, in the posterior cell Spdo localizes at the plasma membrane where it physically interacts with the Notch receptor to specify the Notch-dependent fate. This cell adopts the pIIa fate. We recently isolated the Drosophila AP47 gene encoding the µ1 subunit of the AP-1 clathrin adaptor complex as a novel regulator of Notch signalling in these epithelial cells. We report that loss of function of AP47 causes an early embryonic lethality indicating that AP47 is an essential gene as previously reported in C. elegans and mice. In mosaic flies, loss of function of AP47 causes a pIIb to pIIa cell fate transformation. Similar results were also obtained using tissue specific RNAi against any of the four subunits of the AP-1 complex. The unequal segregation of cell fate determinants is unaffected in cells mutant for AP-1. The steady state distribution of Delta and Notch remain unaffected in AP47 mutant cells. However, we observe that Spdo trafficking is strongly altered. In cells mutant for AP47, Spdo strongly accumulates at the apical surface of the pI cell and that of both pI daughter cells, where it colocalizes with Notch. We propose that AP-1 regulates cell fate by inhibiting the plasma membrane localization of Spdo and thereby preventing the activation of Notch signalling in the anterior pI daughter cell.

2264

Gap Junctional Intercellular Communication and Connexin 43 Are Essential for Maintaining Embryonic Stem Cell Self-Renewal and Pluripotency.

M. Todorova[1], B. Soria[2], I. Quesada[1]; [1]Cellular Physiology and Nutrition, Institute of Bioengineering, Miguel Hernandez University, ELCHE, Spain, [2]Andalusian Center for Molecular Biology and Regenerative Medicine, Seville, Spain

Mouse embryonic stem cells (mESC) derived from blastocysts, are pluripotent and can be maintained in a self-renewing and undifferentiated state in vitro for a long time. Alternatively, under certain conditions they can differentiate into more specific cells. Despite their importance in regenerative medicine and developmental biology, the mechanisms implicated in the maintenance of ESC in a proliferative and pluripotent state are still not fully understood. In this regard, the main objective of this study was to investigate whether the intercellular communication mediated by gap junctions (GJIC) plays a role in these processes. Using the Fluorescence Recovery After Photobleaching (FRAP) technique we detected the expression of functional gap junctions in D3 mESC. More specifically, we established a crucial role for Connexin 43 (Cx43) in this cell-to-cell communication. Its expression was detected by PCR, immunocytochemistry and western blot. Both pharmacological blockers of GJIC and specific Cx43 down-regulation by small interfering RNA (siRNA) caused a profound inhibitory effect on GJIC which led to a loss of their undifferentiated state. This was manifested by dramatic changes in their morphology and a significant decrease in the protein expression of pluripotent markers such as alkaline phosphatase, Oct-3/4 and Nanog. An up-regulation of several differentiation markers like Neurofilament-200 and Glial fibrillary acidic protein was also observed. BrdU incorporation assays also showed a strong decrease in the proliferation rate of Cx43-deficient cells. These cells were viable and not apoptotic as detected by Hoechst staining and viability kits. The lack of GJIC and Cx43 affected also the pluripotency of ESC as under these conditions they were not able to form embryoid bodies. Interestingly, upon withdrawal of the blocking agents this property was restored. Our results outline a major role of GJIC and Cx43 in the regulation of self-renewal and maintenance of pluripotency of ESC. Not only could this data be essential in the understanding of the basic biology of ESC but as well be useful in revealing the early events that occur during embryonic development.

Minisymposium 27: Cellular Basis of Morphogenesis (2265 – 2270)

2265

Polarization of Microtubule Cytoskeleton during Zebrafish Gastrulation.

L. Solnica-Krezel, D. Sepich; Department of Biological Sciences, Vanderbilt University, Nashville, TN

During vertebrate gastrulation convergence and extension movements narrow the embryonic body from back to belly while elongating it from head to tail. We demonstrated that at early zebrafish gastrulation internalized lateral mesodermal cells migrate away from the blastopore, while convergence toward the dorsal midline starts around midgastrulation. Gastrulation movements of lateral and paraxial mesoderm are driven by several distinct cell behaviors. Slow dorsal convergence involves directed migration of moderately elongated cells along complex trajectories. Subsequently, mesodermal cells become mediolaterally elongated and migrate dorsally along straight trajectories with high net speed, and later engaged in polarized cell intercalations. Genetic studies showed that Wnt/Planar Cell Polarity signaling is required for mediolateral cell polarization underlying the transition from slow to fast directed cell migration, and for polarized cell intercalations. The molecular events connecting Wnt/PCP signaling to specific cell behaviors are not clear. Here we address the organization of microtubule cytoskeleton in gastrula cells polarized via Wnt/PCP signaling. To assess polarity of the microtubule cytoskeleton in live zebrafish gastrulae, we label the microtubule organizing center (MTOC) and nucleus with Xcentrin-GFP, the cell membrane with RFP, and collect confocal images. From these images we determine the position of the MTOC relative to the nucleus, direction of movement and mediolateral and anteroposterior embryonics coordinates. We found cells of both the ectoderm and mesoderm have a progressively polarized arrangement of the nucleus-MTOC-leading edge during late gastrulation. Surprisingly, the placement of MTOC is very dynamic; at late

gastrulation MTOC is preferentially located in dorsoposterior cell portion, whereas later it is biased to either dorsal or medial position. By contrast, the orientation of the MTOC is randomized in embryos with defective components of Wnt/PCP signaling. Our studies reveal highly dynamic and Wnt/PCP-dependent polarization of MTOC in cells engaged in gastrulation movements.

2266

Pulsed Actin-Myosin Network Contractions Drive Apical Constriction.

A. Martin[1], M. Kaschube[2], E. Wieschaus[1]; [1]Howard Hughes Medical Institute, Molecular Biology, Princeton University, Princeton, N.J, [2]Lewis-Sigler Institute for Integrative Genomics, Princeton University, Princeton, NJ

Apical constriction facilitates epithelial sheet bending and invagination during morphogenesis. Apical constriction is conventionally thought to be driven by the continuous purse-string-like contraction of a circumferential actin and Non-Muscle Myosin-II (myosin) belt underlying adherens junctions. However, it is unclear whether other force-generating mechanisms can drive this process and how cell-cell interactions influence the actin cytoskeleton. Here, we use real-time imaging and quantitative image analysis of Drosophila gastrulation to show that apical constriction of ventral furrow cells is pulsed. Repeated constrictions, which are asynchronous between neighboring cells, are interrupted by pauses in which the constricted state of the cell apex is maintained. In contrast to the purse-string model, constriction pulses are powered by actin-myosin network contractions that occur at the medial apical cortex and pull discrete adherens junction sites inward. This apical actin-myosin network appears to extend across cell boundaries, forming a web-like meshwork across the constricting epithelium. The transcription factors Twist and Snail differentially regulate pulsed constriction. Expression of Snail controls actin-myosin network contractions while expression of Twist stabilizes the constricted state of the cell apex. Our results suggest a model in which a cortical actin-myosin cytoskeleton functions as a sub-cellular ratchet to incrementally reduce apical area. We are currently investigating how constriction pulses are coordinated across the epithelium and how tensile forces transmitted through the tissue influence cytoskeletal organization and dynamics.

2267

Chemokine Signaling Controls Endodermal Migration during Zebrafish Gastrulation.

T. Schilling, S. Nair; University of California, Irvine, Irvine, CA

Directed cell movements during gastrulation establish the germ layers of the vertebrate embryo and coordinate their contributions to different tissues and organs. Anterior migration of mesoderm and endoderm has largely been interpreted to result from epiboly and convergent-extension movements that drive body elongation. We show that the chemokine cxcl12b and its receptor Cxcr4A restrict anterior migration of endoderm during zebrafish gastrulation, thereby coordinating its movements with mesoderm. Depletion of either gene product causes the endoderm to separate from mesoderm and migrate farther anterior than normal, due to disruption of integrin-dependent cell adhesion. This results in bilateral duplications of endodermal organs, which may have relevance to human organ defects.

2268

Role of Fat-Like Cadherins in Regulation of Planar Polarity and the Organization of Oriented Cell Divisions in the Mammalian Kidney.

S. Saburi[1,2], S. Quaggin[1], E. Fischer[3], I. Hester[1], M. Gessler[1], M. Pontolgio[3], H. McNeill[1,2]; [1]Samuel Lunenfeld Research Institute, University of Toronto, Toronto, ON, Canada, [2]Samuel Lunenfeld Research Institute, Mt. Sinai Hospital, Toronto, ON, Canada, [3]Pasteur Institute, Paris, France

Tissue organization in Drosophila is regulated by the core Planar Cell Polarity (PCP) genes Frizzled, Dishevelled, Prickle, Van Gogh and Flamingo. Core PCP genes are conserved in mammals, and function in mammalian tissue organization. Recent studies have identified another group of Drosophila PCP genes, consisting of the protocadherins Fat and Dachsous, and the transmembrane protein Four-jointed (Fj). In Drosophila, Fat represses Fj transcription, and Ds represses Fat activity in PCP. Fat regulates PCP through the transcriptional co-repressor Atrophin. Here we show that Fat4 is an essential gene that plays a key role in vertebrate PCP. Loss of Fat4 disrupts oriented cell divisions and tubule elongation during kidney development, leading to cystic kidney disease. Fat4 genetically interacts with the PCP genes Vangl2 and Fjx1 in cyst formation. In addition, Fat4 represses Fjx1 expression, indicating Fat signalling is conserved. Together these data suggest that Fat4 regulates vertebrate PCP, and that loss of PCP signalling may underlie some cystic diseases in man. Vertebrates have 4 fat-family cadherins, Fat1-4. To determine the role of these other family members in the planar organization of tissues we are currently examining double mutant combinations of Fat1, Fat4 and Fat3 in the developing mouse kidney, as well as the contribution of Atrophin genes to the development of cystic kidney disease.

2269

Laminin Induces a Switch in the Cytoskeletal and Exocytic Machinery Involved in Neurite Initiation.

S. L. Gupton, F. B. Gertler; Koch Institute for Cancer Research, MIT, Cambridge, MA

Neurite initiation, a process unique to neuronal development that precedes neuronal polarization and axon specification, is driven by actin and microtubule dynamics. We have shown previously that the activity of the Ena/VASP family of actin regulatory proteins is necessary for filopodia formation and subsequently neurite initiation in primary cortical neurons. Plating Ena/VASP-deficient neurons on laminin induces filopodia-like structures and rescues neurite initiation. In this study we aimed to discover the downstream pathways of both Ena/VASP-dependent neurite initiation, as well as laminin-dependent (Ena/VASP independent) neurite initiation. Dynamic microtubules are necessary in both pathways, and thus we hypothesized microtubules deliver membrane/cargo to the plasma membrane via exocytosis at filopodia. To test this hypothesis we used Tetanus Neurotoxin (TeNT) to inactivate the v-SNARE VAMP2 and block VAMP2-mediated exocytosis. TeNT blocked neurite initiation in neurons plated on poly-D-Lysine, suggesting VAMP2-mediated exocytosis is necessary for neurite initiation downstream of Ena/VASP. Surprisingly, in neurons plated on laminin, TeNT cleaved VAMP2 but did not block neurite initiation, indicating laminin-dependent neurite initiation may use alternative exocytic machinery. We utilized function blocking antibodies, pharmacological perturbations, and expression of dominant negative constructs, to determine the downstream pathway of laminin-mediated neurite initiation.

These assays revealed α3β1 and α7β1 integrin function, as well as focal adhesion kinase and Src kinase activity are mandatory. We have subsequently determined that a switch in modes of actin cytoskeleton modulation, from Ena/VASP to the Arp2/3 complex, and in the exocytic machinery, from VAMP2 to VAMP7, is triggered during laminin-dependent neurite initiation. This study reveals exciting alternative pathways for signaling, actin dynamics and exocytosis that drive neurite initiation under different extrinsic conditions.

2270

Functional Modulation of Cardiac Form through Regionally Confined Cell Shape Changes.

D. Yelon; Skirball Institute Developmental Genetics Program, NYU School of Medicine, New York, NY

Developing organs acquire a specific three-dimensional form that insures their normal function. Cardiac function, for example, depends upon properly shaped chambers that emerge from a primitive heart tube. The cellular mechanisms that control chamber shape are not yet understood. Here, we demonstrate that chamber morphology develops via changes in cell morphology, and we determine key regulatory influences on this process. Focusing on the development of the ventricular chamber in zebrafish, we show that cardiomyocyte cell shape changes underlie the formation of characteristic chamber curvatures. In particular, cardiomyocyte elongation occurs within a confined area that forms the ventricular outer curvature. Since cardiac contractility and blood flow begin before chambers emerge, cardiac function has the potential to influence chamber curvature formation. Employing zebrafish mutants with functional deficiencies, we find that blood flow and contractility independently regulate cell shape changes in the emerging ventricle. Reduction of circulation limits the extent of cardiomyocyte elongation; in contrast, disruption of sarcomere formation releases limitations on cardiomyocyte dimensions. Thus, the acquisition of normal cardiomyocyte morphology requires a balance between extrinsic and intrinsic physical forces. Together, these data establish regionally confined cell shape change as a cellular mechanism for chamber emergence and as a link in the relationship between form and function during organ morphogenesis.

Minisymposium 29: Membrane Heterogeneity and Trafficking (2271 – 2276)

2271

Towards a New Paradigm for GTPase Regulation of Intracellular Protein Targeting.

S. Shan, X. Zhang; California Institute of Technology, Pasadena, CA

Two homologous GTPases in the signal recognition particle (SRP) and SRP receptor (SR) control the co-translational targeting of newly synthesized proteins to cellular membranes. SRP and SR represent a growing number of noncanonical GTPases that do not use the classical 'GTPase switch' mechanism. Instead, these GTPases undergo a series of discrete conformational changes when they interact with one another, culminating in reciprocal GTPase activation in the SRP-SR complex. We show that each of the conformational rearrangements during the SRP-SR interaction provides a regulatory point that allow these GTPases to sense and respond to spatial and temporal cues such as cargo binding or membrane localization, thereby ensuring the efficient delivery of cargo proteins to the target membrane, and potentially improves the fidelity of protein targeting via kinetic proofreading mechanisms. Thus the SRP and SR GTPases, despite the absence of a classical 'GTPase switch' and the lack of external regulatory factors, can nevertheless use their intrinsic conformational flexibility to provide exquisite spatial and temporal control of protein targeting.

2272

The GET Complex Mediates the Insertion of Tail Anchored Proteins into the ER Membrane.

M. Schuldiner[1], J. Metz[2], V. Schmid[2], V. Denic[1], M. Rakwalska[2], H. Dieter Schmitt[3], B. Schwappach[2], J. Weissman[1]; [1]CMP, UCSF, San Francisco, CA, [2]Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom, [3]Department of Molecular Biology, MPI for Biophysical Chemistry, Göttingen, Germany

Tail anchored (TA) proteins are functionally diverse polypeptides defined by the presence of a single membrane-anchoring C terminal transmembrane domain. TA proteins are found throughout the secretory pathway, in the nuclear envelope, peroxisomes and mitochondria. Within the secretory pathway, TA proteins play diverse roles such as enabling vesicular traffic (e.g., many of the SNAREs which mediate fusion of secretory vesicles are TA proteins, aiding in protein translocation and promoting folding or degradation of membrane proteins. It has been long known that TA proteins of the secretory pathway, peroxisomes and the nuclear envelope, are first inserted into the the endoplasmic reticulum (ER) membrane and are then sorted to their ultimate destination, yet the cellular machinery responsible for their insertion into proper target membranes remains poorly characterized. I will present both in vivo and in vitro data to show that Get3, the yeast homolog of the mammalian TA-interacting factor Asna1/Trc40, specifically recognizes newly synthesized TA proteins destined for the ER. Get3-TA protein complexes are then recruited to the ER by the Get1/Get2 membrane receptor. In the absence of Get1/Get2, Get3-TA complexes aggregate in the cytosol leading to broad defects in secretory pathway TA protein biogenesis. Thus the GET pathway represents a highly conserved and potentially regulated mechanism by which cells overcome the inherent ambiguity in TA anchor domains preventing their aggregation or insertion into inappropriate membranes.

2273

Structural Basis for the Activation of the Rab Ypt1p by the TRAPP Membrane Tethering Complexes.

Y. Cai[1], H. Chin[2], D. Lazarova[1], S. Menon[1,3], C. Fu[1], H. Cai[1], A. Sclafani[1,3], E. De La Cruz[2], S. Ferro-Novick[1,3], K. Reinisch[1]; [1]Department of Cell Biology, Yale University School of Medicine, New Haven, CT, [2]Molecular Biophysics and Biochemstry, Yale University, New Haven, CT, [3]Cellular and Molecular Medicine, UCSD, La Jolla, CA

The TRAPP membrane tethering complexes mediate vesicle traffic to the Golgi apparatus, with TRAPPI functioning in traffic from the ER to the early Golgi and TRAPPII in traffic between the early endosome and the late Golgi or between Golgi cisternae. TRAPPI also activates the Rab Ypt1p, a step required for membrane fusion to proceed. TRAPPI consists of seven different subunits, of which four (Bet3p, Bet5p, Trs23p, and Trs31p) are essential for guanine nucleotide exchange activity. To understand how the TRAPP subunits cooperate in activating

Ypt1p, we determined the 3.7 Å resolution crystal structure of a heteropentameric TRAPPI subcomplex, which is active as a exchange factor, in complex with the nucleotide-free form of Ypt1p. The complex represents an intermediate in the exchange pathway, after GDP has left the nucleotide binding pocket of Ypt1p and before GTP has bound. Ypt1p is bound primarily to a surface comprising Trs23p but also interacts with Bet5p and with two copies of Bet3p. Via these interactions, the Yp1tp nucleotide binding pocket is stabilized in an "open" conformation that facilitates both nucleotide dissociation and reassociation. Both of the Ypt1p switch regions are reorganized. In switch I, an aromatic residue (Y33) that normally stabilizes bound nucleotide by an edge to face interaction with the guanosine base is disordered, lowering the affinity of TRAPPI-bound Ypt1p for nucleotide. The C terminus of one Bet3p copy invades the Ypt1p nucleotide binding and likely initiates conformational changes in Ypt1p. The Trs31p subunit does not contact Ypt1p in the structure but is required to regulate the surface of Trs23 that interacts with the Rab. TRAPPII contains all TRAPPI subunits required for Ypt1p activation. We show that it also activates Ypt1p, likely by the same mechanism as TRAPPI.

2274

Subunit Organization and Functional Architecture of Vps-C/HOPS and Vps-C/CORVET Complexes.

R. L. Plemel, C. L. Brett, B. L. Lobingier, A. J. Merz; Biochemistry, University of Washington, Seattle, WA

Membrane traffic through the terminal compartments of the late endosomal system is coordinated by Rab5 and Rab7 family GTPases, which act in concert with a set of overlapping ~700 kDa protein complexes, HOPS and CORVET. Each of these complexes contains four Vps-C proteins (Vps11/16/18/33). Each complex also contains two complex-specific subunits (Vps39/41 or Vps3/8). We show that each complex contains at least two Rab GTPase binding sites, and we present a domain-level map of subunit associations among all of the HOPS and CORVET subunits. Finally, we present biochemical and genetic analyses suggesting that a specific ~200 residue domain within the Vps11 subunit serves as a nexus for integration of Rab signaling, membrane tethering, and fusion regulation, for both the HOPS and CORVET Vps-C complexes.

2275

Golgi Phosphoinositides and Growth Control of Secretion.

A. Blagoveshchenskaya, F. Cheong, P. Mayinger; Oregon Health & Science University, Portland, OR

Cell growth requires proportional delivery of lipids and proteins to the periphery via the secretory pathway. How secretion is coordinated with growth is not known. The objective of this work was to identify and characterize the missing functional link between cell growth and the secretory pathway. We found that the lipid phosphatase Sac1 regulates Golgi phosphatidylinositol-4-phosphate (PI(4)P) in a growth-specific manner. In turn, the rate of anterograde membrane traffic from the Golgi depends on the levels of PI(4)P. In yeast, the intracellular distribution of Sac1 phosphatase is regulated by nutrients, whereas mammalian Sac1 orthologs respond to the levels of growth factors (1,2). Human SAC1 oligomerizes during serum-starvation and accumulates at the Golgi, where it down-regulates anterograde trafficking by depleting PI(4)P levels. When quiescent cells are stimulated by mitogens, the majority of SAC1 shuttles back to the ER, thus releasing the brake on Golgi secretion. We discovered that the p38 mitogen-activated kinase (MAPK) pathway is required for the rapid Golgi-to-ER translocation of SAC1. p38 activity is necessary for dissociating SAC1 oligomers after mitogen stimulation, which triggers retrograde trafficking of SAC1 to the ER. These results suggest direct roles for p38 MAPK and SAC1 in transmitting growth signals to the secretory machinery. In proliferating cells a certain proportion of SAC1 remains associated with the Golgi and maintains a trans-to-cis gradient of PI(4)P, which is critical for proper Golgi enzyme localization. We conclude that SAC1-dependent control of Golgi phosphoinositides is important for the enhanced membrane traffic that is needed for rapid cell proliferation but also plays a house-keeping role in the structural organization of the Golgi. 1. Faulhammer, F., Kanjilal-Kolar, S., Knodler, A., Lo, J., Lee, Y., Konrad, G., and Mayinger, P. (2007) Traffic 8, 1554-1567 2. Blagoveshchenskaya, A., Cheong, F. Y., Rohde, H. M., Glover, G., Knodler, A., Nicolson, T., Boehmelt, G., and Mayinger, P. (2008) J Cell Biol 180, 803-812

2276

Exo70 Interacts with Phosphatidylinositol 4,5-Bisphosphate and Mediates the Targeting of the Exocyst to the Plasma Membrane for Exocytosis.

B. He, J. Liu, W. Guo; Department of Biology, University of Pennsylvania, Philadelphia, PA

The exocyst is an octameric protein complex implicated in the tethering of post-Golgi secretory vesicles to the plasma membrane prior to fusion. To understand the mechanism of vesicle tethering, it is important to elucidate how the tethering complex itself associates with the target membrane. Here we report that Exo70 plays a pivotal role in the targeting of the exocyst complex to the plasma membrane. Both yeast and human Exo70 interact directly with phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) through conserved residues located at their C-termini. This interaction is specific to $PI(4,5)P_2$ as Exo70 shows little or no interaction with the stereoisomeric $PI(3,5)P_2$ or monophosphorylated phosphoinositides such as PI(3)P and PI(4)P, which are localized to other membrane compartments such as endosomes and Golgi. In yeast, the association of the exocyst complex with the plasma membrane was diminished in the temperature-sensitive *mss4* mutant cells in which the $PI(4,5)P_2$ level in the plasma membrane was reduced. Furthermore, disruption of the exocyst-$PI(4,5)P_2$ interaction inhibits polarized exocytosis at the daughter cell membrane. In Hela cells, disruption of the exocyst-$PI(4,5)P_2$ interaction inhibits the docking and fusion, but not the delivery, of secretory vesicles carrying VSV-G to the plasma membrane. We propose that an evolutionarily conserved mechanism involving a direct interaction between Exo70 and $PI(4,5)P_2$ mediates the targeting of the exocyst complex to the plasma membrane for exocytosis. The interactions of specific tethering proteins with distinct sets of Rab GTPases and phosphoinositides may be crucial for membrane traffic fidelity and organelle identity.

Minisymposium 30: Non-Coding RNAs (2277 – 2282)

2277

Regulation of Gene Expression by Micrornas in C. Elegans.

A. Pasquinelli; University of California, San Diego, La Jolla, CA

MicroRNAs (miRNAs) comprise a new class of small regulatory RNAs that inhibit the expression of protein-coding target genes. There are hundreds, perhaps thousands of miRNAs encoded by the human genome and already mis-expression of specific miRNA genes has been linked abnormal development and disease. Since the recent emergence of this family of regulatory RNAs, rapid progress has been made in identifying new miRNAs and in determining factors important for their expression and function. However, the mechanism by which these regulatory RNAs repress specific target mRNAs is still unclear. My lab primarily focuses on the founding miRNA genes, lin-4 and let-7, and their genetically defined targets in C. elegans to understand how these miRNAs control development of the animal. The majority of animal miRNAs, including lin-4 and let-7, recognize sites of partial complementarity in the 3' untranslated regions (UTRs) of target genes, and this typically results in translational repression and mRNA degradation. Currently, we are exploring the generality of mRNA degradation as an outcome of regulation by miRNAs. We are also attempting to identify the mRNA degradation intermediates and the protein factors that participate in this mechanism of regulation by miRNAs. Studying miRNAs and their targets in C. elegans allows us to analyze the effect of authentic mutants in miRNA genes on endogenous gene expression. By elucidating the function of miRNAs in this organism, we hope to contribute to the general understanding of this newly discovered and widely conserved mode of gene regulation.

2278

Switching from Repression to Activation: MicroRNAs Can Upregulate Translation.

S. Vasudevan, J. Steitz; MBB, Yale University, New Haven, CT

AU-rich elements (AREs) and microRNA target sites are conserved sequences in mRNA 3′-untranslated regions (3′UTRs) that post-transcriptionally control gene expression. Upon G0 cell-cycle arrest in human cell lines, the ARE in tumor necrosis factor α (TNFα) mRNA is transformed into a translation activation signal, recruiting microRNP-associated factors, Argonaute (AGO) and fragile-X-mental-retardation-related protein 1 (FXR1). We demonstrate that human microRNA miR369-3p directs association of these proteins with the ARE to activate translation. Furthermore, we document that two well-studied microRNAs—Let-7 and the synthetic microRNA cxcr4—likewise induce translation upregulation of target mRNAs upon G0 cell-cycle arrest; yet, they repress translation in proliferating cells. Thus, activation can be a common function of microRNPs upon G0 cell-cycle arrest or quiescence. In contrast, we find that siRNAs, unlike microRNAs, downregulate expression of a reporter in serum-starvation-induced G0 arrested cells, as well as in proliferating cells. We propose that translation regulation by microRNPs oscillates between repression and activation during the cell cycle. We observe the strongest repressive potential in the S and S/G2 phases with minimal repression in the G1 phase. Importantly, we report activation in naturally quiescent systems, reaffirming that the quiescent state and not serum-starvation-induced stress causes microRNA-mediated translation upregulation. Our data underscore the importance of the quiescent state for microRNA-mediated translation activation and suggest the potential for further novel microRNA-mediated functions in distinct cell fates.

2279

Micrornas Regulate the G1/S Transition of Embryonic Stem Cells.

Y. Wang[1], S. Baskerville[2], A. Shenoy[1], J. Babiarz[1], L. Baehner[1], R. Blelloch[1]; [1]UCSF, San Francisco, CA, [2]Dharmacon Technologies, Lafayette, CO

Embryonic Stem (ES) cells have a rapid cell cycle with a short G1 phase. The mechanisms underlying their unique cell cycle structure remain largely unknown. Recently, we generated a Dgcr8 knockout model to globally remove microRNAs (miRNA) in mouse ES cells. These miRNA depleted ES cells showed a decreased proliferation rate with an associated accumulation of cells in the G1 phase of the cell cycle, implicating important roles of miRNAs in regulating the ES cell cycle. To identify the specific miRNAs, we designed a screening strategy in which individual miRNA mimics were reintroduced into Dgcr8 knockout ES cells and evaluated for rescue of the proliferation and cell cycle defects. A library of 266 known mouse miRNAs was screened. Fourteen of these miRNAs dramatically improved Dgcr8 knockout ES cell proliferation. Mimics for ten of these miRNAs were resynthesized and further evaluated. These ten miRNAs reduced the fraction of DGCR8 knockout cells in the G1 phase. In contrast, these same miRNAs had no effect on the proliferation or cell cycle of wild-type ES cells, suggesting they already exist at saturating levels in ES cells. Consistent with this, five of the miRNAs are highly expressed in undifferentiated ES cells. Interestingly, these miRNAs share a similar seed sequence. Therefore, they likely regulate common targets. Western analysis and luciferase reporter assays identified several key regulators of the G1/S transition targeted by these miRNAs. Our results show that miRNAs play an important role in expediting the G1/S transition. A shortened cell cycle is critical during early mammalian embryogenesis allowing the young embryo to rapidly grow prior to somatic differentiation. The large number of miRNAs found in our screen suggests a large degree of redundancy in this critical cellular function. Inappropriate expression of these miRNAs in differentiated cells could lead to abnormally rapid cell growth. Therefore, these same or related miRNAs may support cellular transformation and associated tumor growth. Our ongoing studies are aimed at testing these hypotheses as well as searching for other potential cell cycle targets of these miRNAs.

2280

Mtor Regulates Skeletal Muscle Growth through Microrna Mir-1 and Follistatin.

Y. Sun[1], Y. Ge[1], M. Band[2], J. Drnevich[2], J. Chen[1]; [1]Department of Cell and Developmental Biology, University of Illinois at Urbana-Champaign, Urbana, Urbana, IL, [2]W. M. Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign, Urbana, IL

The mammalian target of rapamycin (mTOR) is a Ser/Thr kinase that governs a myriad of cellular and developmental processes. Previously we have shown that skeletal muscle development requires mTOR signaling at two distinct stages, and that mTOR controls the expression of a yet-to-be-identified secreted factor critical for myotube growth. In recent years microRNA has emerged as an important regulatory mechanism in skeletal myogenesis. To probe potential connections between mTOR signaling and microRNA in the regulation of myogenesis, we performed microRNA expression profiling with differentiating C2C12 cells, and found several microRNAs that were significantly

affected by the specific mTOR inhibitor rapamycin in expression. Among those, miR-1 was up-regulated during differentiation and this increase was markedly blocked by rapamycin in both C2C12 and mouse primary myocytes. We further discovered that mTOR regulates the transcription of miR-1 through its upstream enhancers, and that this regulation requires mTOR kinase activity and is at least partially mediated by the myogenic transcription factor MyoD. To delineate the pathway downstream of mTOR-miR-1 in myogenesis, we considered the reported miR-1 target HDAC4, and the HDAC inhibitor-inducible myogenic factor follistatin. We hypothesized that miR-1, HDAC4 and follistatin may act in a linear pathway to mediate mTOR signaling, and that follistatin may be the long-sought fusion factor regulated by mTOR in muscle growth. This model is now fully supported by these observations: (1) anti-miR-1 (LNA) up-regulated HDAC4 expression and down-regulated follistatin expression; (2) rapamycin phenocopied anti-miR-1 in the regulation of HDAC4 and follistatin; (3) anti-miR-1 inhibited myotube growth, which was rescued by recombinant follistatin, and by the HDAC inhibitor trichostatin A (TSA); (4) rapamycin inhibition of myotube growth was reversed by recombinant follistatin, as well as by TSA. Taken together, our findings reveal for the first time a link between mTOR signaling and microRNA, and identify a novel mTOR-miR-1-HDAC4-follistatin pathway that regulates a late fusion stage critical for myotube maturation and skeletal muscle growth.

2281

Upregulation of Mir-196 during Early Stages of Blastema Formation Is Essential for Tail Regeneration in the Axolotl.

T. Sehm[1], C. Sachse[2], K. Echeverri[1]; [1]Center for Regenerative Therapies, Dresden, Germany, [2]Cenix Bioscience, Dresden, Germany

Salamanders are among the few organisms fortunate in their ability to regenerate many body parts in response to major injury. Despite the obvious medical potential, our detailed understanding of the underlying molecular pathways remains largely in its infancy. As microRNAs have emerged in recent years as key regulators of gene expression underlying a wide range of cellular differentiation "programs" in many species, we have undertaken the study of miRNA expression and function during axolotl regeneration. As a first step, we conducted a miRNA expression profiling analysis using microarrays probing known vertebrate miRNAs, to determine whether any of these are detectable and differentially modulated early during axolotl tail regeneration. Among the 79 conserved miRNAs thereby found to exhibit significantly altered expression patterns in the 3-day regenerating tail, miR-196 was notable both as one of the most highly up-regulated miRNAs (32-fold vs mature tissue), and by its previous implication in chick and mouse limb development. We therefore examined the role of miR-196 further by specifically blocking its expression in the regenerating tail through microinjection of a synthetic "antagomir" inhibitor. This treatment specifically inhibited tail regeneration, confirming the essential role of miR-196 in this process. More detailed phenotypic analyses revealed that miR-196 inhibition results in significantly altered expression boundaries for Pax7 protein in the spinal cord, thereby identifying miR-196 as a key regulator of patterning during tail regeneration. Down-regulation of miR-196 results in an inhibition of regeneration due to mis-patterning within the spinal cord that appears to alter the dorsal boundaries within the spinal cord and also the overall anterior/posterior patterning information within the cells at the amputation site. This data identifies miR-196 as a key regulator of patterning within the spinal cord by regulating the levels of BMP, Msx and Pax7 during regeneration. Ongoing experiments are revealing the identities and roles of other molecules acting as intermediates, effectors and regulators within this newly-emerging pathway.

2282

The Role of Micrornas during Muscle Development: Analysis of the Muscle Microrna Targetome.

A. Giraldez; Department of Genetics, Yale University, New Haven, CT

MicroRNAs comprise ~4% of all vertebrate genes, with potential to regulate more than one third of the protein coding genes, but their in vivo functions and mechanisms of action remain largely unknown. To block all miRNA formation in zebrafish, we generated maternal-zygotic dicer (MZdicer) mutants that disrupt the Dicer ribonuclease III and double-stranded RNA-binding domains. In previous studies we have shown that microRNAs repress translation and accelerate target mRNA degradation. To identify the muscle microRNA targetome in vivo, we have analyzed the mRNA expression profile of GFP labeled muscle cells after sorting. We have identified several hundred mRNAs upregulated in muscle cells in the absence of miRNAs (MZdicer and single miRNA knock down) compared to wild type muscle cells. Using this approach we have identified several hundred miRNA targets in muscle development. Identification of miRNA target sites in the genes upregulated in the absence of miRNAs revealed 10-fold enrichment for miR-1 and miR-133 complementary sequences. Target validation and in situ analysis suggest that microRNAs regulate muscle homeostasis and shape gene expression in the muscle by downregulating post-transcriptionally a large fraction of genes during development. These results provide the first genome wide analysis of miRNA targets in vivo during organogenesis in a vertebrate embryo revealing a widespread impact of microRNAs in gene expression. We are currently analyzing the role of muscle miRNAs to buffer genetic and environmental perturbations during embryogenesis. In this meeting we will present our functional analysis of the miRNA targets in muscle development.

Minisymposium 31: Organelle Biogenesis and Turnover (2283 – 2288)

2283

The Selective Degradation of Mitochondria by Autophagy.

D. Klionsky, T. Kanki; Life Sciences Institute, Dept of Molecular, Cellular and Developmental Biology, Dept of Biological Chemistry, University of Michigan, Ann Arbor, MI

Autophagy is a ubiquitous process used for subcellular degradation and cellular remodeling. During macroautophagy, a cytosolic double-membrane vesicle sequesters a portion of the cytoplasm, including entire organelles, and delivers this cargo to the lysosome (the vacuole in yeast) for subsequent breakdown and recycling of the resulting macromolecules. Autophagy plays an important role during conditions of nutrient limitation, but it also functions in tumor suppression, the immune response, and the prevention of certain types of neurodegeneration. Over thirty genes have been identified in fungi that are specific to autophagy-related pathways, and homologs for many of these genes have been identified in higher eukaryotes. A subset of the corresponding gene products is required for selective types of autophagy including the cytoplasm to vacuole targeting (Cvt) pathway and the specific degradation of superfluous peroxisomes, termed pexophagy. Mitochondria can

also be degraded by autophagy, and it is proposed that this can occur through a selective mechanism. For example, targeting damaged mitochondria for degradation can prevent nuclear DNA mutations resulting from excess reactive oxygen species, and can also prevent the need for apoptotic cell death; however, there has been little experimental evidence of selective mitochondrial degradation, or mitophagy. We developed a new system to monitor mitochondrial degradation and used it to examine mutant strains that are defective in specific types of autophagy to determine whether there is a mechanism to selectively degrade this organelle. For example, Atg11 acts as a scaffold to link various components of the autophagic machinery to the cargo of the Cvt pathway; Atg11 is required for import of the Cvt cargo protein precursor aminopeptidase I, but is not needed for non-selective autophagy. Similarly, Atg11 is essential for pexophagy, again serving as an adaptor between the cargo and the sequestering vesicle that delivers the organelle to the vacuole. We find that Atg11 and other proteins needed for selective autophagic processes are required for the degradation of mitochondria, confirming the presence of selective mitophagy.

2284

Molecular Mechanisms Controlling Bacterial Membrane-Bound Organelle Formation.

D. Murat[1], H. Vali[2], A. Komeili[1]; [1]Plant and Microbial Biology Department, University of California Berkeley, Berkeley, CA, [2]Anatomy and Cell Biology, McGill University, Montreal, QC, Canada

Within the past decade, it has become clear that bacteria are highly organized cells and more than just a "bag of enzymes." Bacteria not only use a eukaryotic-like cytoskeleton to control cell shape, polarity and cell division, but they can also contain membranous compartments reminiscent of eukaryotic organelles. We are using the magnetosomes of magnetotactic bacteria, one of the few examples of bacterial membrane-bound organelles, as a model to study the cell biology of bacterial subcellular compartments. Magnetosomes are formed by invagination of the inner membrane and filled with nanometer-sized magnetite crystals. They are aligned in a chain within the cell, allowing it to orient in magnetic fields. After the magnetosome membrane is formed, a crystal of magnetite is synthesized in each compartment. We are particularly interested in understanding the mechanisms controlling the formation of the magnetosome membrane. In our model organism Magnetospirillum magneticum AMB-1, we know that at least some of the proteins required for magnetosome membrane biogenesis are encoded within a 98 kb genomic island called the Magnetosome Island (MAI). We have been investigating the role of the MAI genes in magnetosome membrane formation by a systematic genetic approach. We have generated large deletions in the MAI and looked for mutants affected in magnetosome membrane biogenesis. We have identified an 18-gene operon which is essential for magnetosome membrane formation. By generating polar and non-polar mutations in that region, we found four genes, predicted to encode membrane proteins, which are required for magnetosome membrane formation. When deleted, three of those four genes affect the way the inner membrane is deformed. However, the deletion of the fourth gene completely prevents any rearrangements of the inner membrane. We are currently investigating whether those four proteins are sufficient to trigger inner membrane deformation in vivo and in vitro. Given that three of the four proteins are only found in bacteria able to make magnetosomes, this suggests that magnetosome membrane formation could potentially rely on a new mechanism for the control of membrane curvature in bacteria.

2285

The Ubiquitin Conjugation System Is Involved in the Disassembly of Cilia/Flagella.

K. Huang, D. Diener, J. Rosenbaum; Department of Molecular Cell & Developmental Biology, Yale University, New Haven, CT

The assembly and maintenance of cilia/flagella are dependent on intraflagellar transport (IFT) and are linked to the cell cycle and environmental cues. Likewise, flagellar disassembly is tied to the cell cycle and is an important facet of flagellar biology. We have found that ubiquitination of flagellar proteins is an integral part of flagellar disassembly. Ubiquitin, ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3) are present in proteomic databases of cilia/flagella. Indeed, we have detected free ubiquitin in flagella and have found that several flagellar proteins were ubiquitinated in isolated flagella when HA-ubiquitin and ATP were added. These data suggest that the ubiquitin conjugation system operates in flagella. Ubiquitinated flagellar proteins increased during flagellar resorption that follows gamete fusion or when resorption is induced by NaPPi or IBMX. This increase was dramatically enhanced during flagellar resorption in temperature sensitive IFT anterograde mutants at the restrictive temperature and in retrograde IFT mutants, suggesting that disassembly (turnover) products are labeled with ubiquitin and transported to the cell body by IFT. Substrates of the ubiquitin conjugation system include α-tubulin (but not β-tubulin), a dynein subunit (IC69), and a signaling protein in the mating process (CrPKG). In addition, over-expression of mutant ubiquitin or inhibiting E1 activity affected both flagellar resorption, and regeneration. The latter effect may be due to an interaction between the disassembly and assembly at the tip of the growing flagella. Our working model is that turnover products of cilia/flagella are conjugated to ubiquitin and transferred to the cell body by IFT. Whether/how ubiquitination controls flagellar turnover and disassembly is under investigation. Supported by NIH grant GM14642 to JLR, and a PKD grant (159a2f) to KH.

2286

Two Novel Beclin 1-Interacting Proteins Distinctly Regulate Vps34 Phosphatidylinositol 3-Kinase Activity and Autophagy.

Q. Wang[1,3], Y. Zhong[2], X. Li[3], N. Heintz[2], B. T. Chait[1], Z. Yue[3]; [1]Laboratory of Mass Spectrometry and Gaseous Ion Chemistry, The Rockefeller University, New York, NY, [2]Laboratory of Molecular Biology, The Rockefeller University, New York, NY, [3]Departments of Neurology and Neuroscience, Mount Sinai School of Medicine, New York, NY

The objective of this work is to uncover novel components of the mammalian autophagy pathway. Among the few mammalian autophagy genes identified to date, *beclin 1* plays important roles in viral defense, tumor suppression, neurodegeneration, development and ageing. However, the molecular mechanism by which Beclin 1 mediates autophagy remains largely unknown. Here we explored autophagy using a BAC transgenic strategy to insert *beclin 1*-EGFP into the genome of a *beclin 1*-knockout mouse. This strategy ensures *beclin 1*-EGFP expression and Beclin 1-EGFP complex formation under physiological conditions. We purified Beclin 1-EGFP complexes from liver, brain and thymus of these mice using an anti-GFP antibody and identified components of the isolated complexes by mass spectrometry. These complexes and their cellular functions were investigated using gel filtration, co-immunoprecipitation, co-localization, protein-protein interaction domain analysis, over-expression and RNAi knock-down, (immuno-)electron microscopy, as well as phosphatidylinositol 3-phosphate (PI(3)P) kinase, liposome binding and endocytosis assays. We show that Beclin 1-EGFP is expressed in the *beclin 1*-EGFP$^{+/-}$

;*beclin 1*$^{-/-}$ mice at nearly endogenous level and completely rescues the embryonic lethality of *beclin 1* homozygous deletion (*beclin 1*$^{-/-}$) mice. Using these "rescued" mice, we identified a core Beclin 1 protein complex which is composed of Beclin 1, UVRAG, Vps34 and Vps15. We also identified two novel Beclin 1-interacting proteins - BISC and BIRF. BISC and Beclin 1 colocalize in large membrane-wrapped structures, together with Atg5 and Atg12. In contrast, BIRF is present in distinct vesicular structures that are Lamp 1-positive. Moreover, RNAi knock-down of BISC and BIRF respectively reduces and enhances autophagic activity. Furthermore, binding of BISC and BIRF to Vps34 respectively enhances and reduces the PI(3)P kinase activity. We hypothesize that regulation of autophagic activity by BISC and BIRF is mediated via Vps34 PI(3)P kinase activity - with BISC regulating early steps of autophagosome formation and BIRF regulating later steps of lysosomal fusion/degradation.

2287

Mdv1 Acts to Nucleate the Assembly of the Mitochondrial Division Dynamin.

L. Lackner, J. Horner, J. Nunnari; Department of Molecular and Cellular Biology, University of California, Davis, Davis, CA

Dynamin related proteins (DRPs) are large GTPases that regulate membrane dynamics in a variety of cellular processes. In S. cerevisiae, the DRP Dnm1 is required for mitochondrial division. We have postulated a mechanistic model of Dnm1-mediated mitochondrial division where, after Dnm1 is targeted to mitochondria, it undergoes GTP-driven assembly into helical structures, which drives membrane constriction. In turn, self-assembly also stimulates GTP hydrolysis, which is likely to evoke conformational changes within the Dnm1 helix that complete membrane fission. Two additional proteins, Mdv1 and Fis1 are required for Dnm1-mediated mitochondrial division and function to target Dnm1 to the mitochondrial surface. Our previous studies suggest that Mdv1 also acts post-targeting with Dnm1 to facilitate division. We determined the post-targeting role of Mdv1 in Dnm1 division complex assembly using pure protein assays to directly assess how the Dnm1-Mdv1 interaction is regulated and how this interaction affects the kinetic and structural properties of Dnm1. Our studies demonstrate that Mdv1 interacts and co-assembles preferentially with the GTP-bound form of Dnm1. This regulated interaction serves to nucleate Dnm1 self-assembly and, therefore, suggests that Mdv1 acts at the early stages of Dnm1 assembly to drive the formation of higher-ordered division competent structures.

2288

Oxysterol-Binding Proteins Transfer Lipids at Organelle Contact Sites.

T. Schulz, M. Choi, J. Mears, J. Hinshaw, W. A. Prinz; NIH/NIDDK, Bethesda, MD

The exchange of small molecules and signals between organelles is thought to occur at regions of membrane apposition called membrane contact sites (MCSs). However, few components of MCSs have been identified and little is known about how they function. The oxysterol-binding protein (OSBP)-related proteins (ORPs) are a large family of conserved lipid-binding proteins implicated in sterol metabolism, vesicular trafficking, and nonvesicular sterol transport. We demonstrate that the ORPs in S. cerevisiae bind both membranes at a MCS and transfer sterols between the bilayers by a novel mechanism. We show that the core lipid-binding domain present in all ORPs has two membrane-binding faces, allowing them to bind two membranes simultaneously and bring them into close apposition. Remarkably, ORPs can transfer sterols between two membranes even while tightly bound to both, suggesting that ORPs can transport lipids without dissociating from membranes. The two membrane binding faces work cooperatively to transport lipid; ORP binding to phosphoinositides in one membrane significantly alters the rate of sterol extraction and transfer from a second. Consistent with a role for ORPs at MCSs, we show that most of the seven ORPs in yeast are enriched at these regions, particularly between the endoplasmic reticulum and plasma membrane. By bridging organelle membranes at MCSs the ORPs are positioned to sense and regulate the lipid content of both membranes.

Minisymposium 32: Stress Responses (2289 – 2294)

2289

ER Stress, Oxidative Stress, and the Unfolded Protein Response.

R. Kaufman; [1]Howard Hughes Medical Institute, Chevy Chase, MD, [2]Department of Biological Chemistry, University of Michigan Medical School, Ann Arbor, MI, [3]Department of Internal Medicine, University of Michigan Medical School, Ann Arbor, MI

The endoplasmic reticulum (ER) is a cellular compartment specialized for chaperone-assisted folding and post-translational modification of nascent polypeptides. Disruption of ER homeostasis through increased biosynthetic load, reduction in calcium storage, energy depletion, or expression of folding incompetent proteins leads to accumulation of unfolded protein and activation of the unfolded protein response (UPR). The UPR emanates from the ER through activation of three transmembrane sensors, IRE1, PERK, and ATF6. IRE1 is a protein kinase with endoribonuclease (RNase) activity that initiates a site-specific unconventional splicing reaction to remove a 26b intron within the XBP1 mRNA. Spliced XBP1 mRNA produces a potent basic leucine zipper (bZiP)-containing transcription factor of the ATF/CREB family that activates UPR genes. Activation of PERK by ER stress leads to inhibition of translation initiation through phosphorylation of eukaryotic initiation factor 2 (eIF2) on the alpha subunit. Paradoxically, several mRNAs require eIF2α phosphorylation for efficient translation, ie. ATF4 mRNA. ATF4 is a transcription factor that activates genes involved in amino acid biosynthesis and transport, anti-oxidative stress responses, and proapoptotic functions, such as CHOP. Finally, ATF6 is a type II ER transmembrane protein that contains a bZiP domain in the cytosol and a stress-sensing domain in the ER lumen. Upon accumulation of unfolded proteins in the ER lumen, ATF6 transits to the Golgi compartment where it is cleaved by the proteases S1P and S2P to generate the cytosolic fragment that traffics to the nucleus to activate transcription of a subset of UPR genes. Studies will be summarized that demonstrate unique and essential features of the cellular response to ER stress and how ER stress is intimately coupled with oxidative stress. For example, glucose-regulated insulin production in pancreatic beta cells requires an intact PERK/eIF2α subpathway to prevent oxidative stress. Studies will show how mouse models can be used to dissect the roles of the UPR subpathways in different physiological and pathological conditions.

2290

Mrna Targeting to ER Stress Signaling Sites.

T. Aragon, E. van Anken, D. Pincus, I. M. Serafimova, A. V. Korennykh, C. A. Rubio, P. Walter; Howard Hughes Medical Institute, and Biochemistry and Biophysics, University of California San Francisco, San Francisco, CA

The accumulation of misfolded proteins in the lumen of the endoplasmic reticulum (ER) is sensed the bifunctional transmembrane kinase/endoribonuclease Ire1 that initiates the unfolded protein response (UPR). The UPR is relayed via a non conventional mRNA splicing reaction. Activated Ire1 catalyzes the excision of an intron from HAC1 mRNA. Translation of the spliced HAC1 mRNA, activates transcription of UPR target genes to adjust the ER protein folding capacity according to need. We show that upon activation, Ire1 molecules cluster in the ER membrane into discrete foci of higher-order oligomers. HAC1 mRNA is recruited to the Ire1 foci by means of a conserved bipartite targeting element contained in the 3' untranslated region (3'UTR) of HAC1 mRNA. Disruption of either Ire1 clustering or of HAC1 mRNA recruitment impairs UPR signaling. The HAC1 3'UTR element is sufficient to target heterologous mRNAs to Ire1 foci, as long as its translation is repressed. Translational repression afforded by the intron fulfills this requirement for HAC1 mRNA. Recruitment of mRNA to signaling centers provides a new paradigm for the control of eukaryotic gene expression.

2291

Ubiquilin and Erasin Regulate ER Stress and Lifespan in Caenorhabditis Elegans.

P. Lim, M. J. Monteiro; UMBI, Baltimore, MD

An accumulation of misfolded proteins in cells and tissues is associated with a number of human diseases, particularly age-related diseases. It is not surprising that cells have therefore evolved a number of quality control systems to monitor and maintain fidelity of protein folding, a sophisticated set of which is present in the endoplasmic reticulum (ER), the organelle where over one-third of all proteins are synthesized. An accumulation of misfolded proteins in the ER activates a series of signaling pathways collectively called the unfolded protein response (UPR) that function to eliminate the misfolded proteins and to restore protein homeostasis. One set of genes that are regulated during UPR are those involved in ER-associated degradation (ERAD), the process by which misfolded proteins in the ER are retrotranslocated from the ER to the cytosol or nucleoplasm for degradation by the proteasomes. Inefficient clearance of misfolded proteins for the ER leads to ER stress, which appears to be involved in many human diseases. We have identified ubiquilin and erasin as components of a novel complex involved in ERAD. Using Caenorhabditis elegans as a model system, we demonstrate that ubiquilin and erasin are UPR genes whose expression are upregulated during tunicamycin-induced ER stress and that the upregulation requires the IRE-1 branch of the UPR. Loss of ubiquilin or erasin by RNA interference (RNAi) resulted in activation of the UPR and increased accumulation of polyubiquitinated proteins in worms. Moreover, loss of ubiquilin or erasin shortened lifespan in worms. We also demonstrate the involvement of ubiquilin in a worm model of Huntington's disease. We show that overexpression of mRFP-tagged ubiquilin prevented and rescued a motility defect caused by expression of human huntingtin exon-1 fragment containing toxic polyglutamine tracts while loss of ubiquilin by RNAi exacerbated this defect. Our results strongly support a role for ubiquilin and erasin as key components in the ER stress response to maintain protein homeostasis.

2292

SREBP Controls Adaptation to Hypoxic Stress in Fission Yeast.

P. Espenshade, B. Hughes, C. S. Lee; Department of Cell Biology, Johns Hopkins University SOM, Baltimore, MD

Eukaryotic cells, from unicellular organisms to cells in mammalian tissues, experience fluctuating environmental oxygen concentrations. In order to adapt to these changes in cellular oxygen supply, cells must detect changes in oxygen concentration and elicit a physiological response to allow the organism or tissue to adapt. Sre1, the fission yeast Sterol Regulatory Element Binding Protein (SREBP), is an ER membrane-bound transcription factor that responds to changes in oxygen-dependent sterol synthesis as an indirect measure of oxygen availability. Under low oxygen, Sre1 is proteolytically cleaved and the released N-terminal transcription factor (Sre1N) activates gene expression essential for hypoxic growth. Here, we report a second oxygen-dependent mechanism for regulation of Sre1 that is independent of sterol-regulated proteolysis. Using yeast expressing only Sre1N, we show that Sre1N turnover is regulated by oxygen. Ofd1, an uncharacterized prolyl 4-hydroxylase-like 2-oxoglutarate-Fe(II) dioxygenase, accelerates Sre1N degradation in the presence of oxygen. However, unlike the prolyl 4-hydroxylases that regulate mammalian hypoxia inducible factor (HIF), Ofd1 uses multiple domains to regulate Sre1N degradation by oxygen; the Ofd1 N-terminal dioxygenase domain is required for oxygen sensing and the Ofd1 C-terminal domain accelerates Sre1N degradation. Our data support a model whereby the Ofd1 N-terminal dioxygenase domain is an oxygen sensor that regulates activity of the C-terminal degradation domain. Thus, oxygen regulates Sre1 activity at two points to mediate adaptation to hypoxic stress: (1) proteolytic cleavage and (2) transcription factor degradation.

2293

Induction of Pleiotropic Drug Resistance by the Molecular Chaperone Zuo1.

A. Prunuske, J. Waltner, E. Craig; University of Wisconsin, Madison, WI

In yeast, the ribosome associated molecular chaperones Zuo1 and Ssz1 (Pdr13) form a complex that is important for folding nascent polypeptides. When these chaperones are free from the ribosome, they are able to induce pleiotropic drug resistance (PDR). During PDR, there is an upregulation of ABC transporters which are then able to pump drugs out of the cell decreasing their toxic effects. This mechanism also occurs in cancer cells that develop multidrug resistance. In protein folding, the J-domain of Zuo1 acts by stimulating the ATPase activity of the Hsp70 Ssb1/2 to promote the association of Ssb1/2 with hydrophobic regions on nascent polypeptides. The role of Zuo1 in PDR appears to be distinct from its role in protein folding since the activation of PDR by Zuo1 does not require its J-domain or Ssb1/2. We have shown that overexpression of the 68 amino acids from the C-terminus of Zuo1 specifically and rapidly upregulates PDR target genes. Within this domain, we have identified a gain of function mutation that increases the upregulation of ABC transporters and acts through the Pdr1 transcription factor and not through its homolog Pdr3. Drugs themselves can induce PDR by binding to Pdr1 and Pdr3 and subsequently recruiting the Mediator subunit Gal11. We have found that the induction of PDR by drug does not require Zuo1 or Ssz1. Instead, the role for

Zuo1 in PDR activation may be to couple protein folding or translation to membrane biogenesis since many of the target genes induced by PDR are involved in phospholipid synthesis.

2294

DNA Zip Codes: An Ancient Mechanism for Gene Targeting to the Nuclear Periphery.

S. Ahmed[1], D. Brickner[1], M. McDonough[1,2], T. Volpe[2], J. Brickner[1]; [1]Dept. of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, Evanston, IL, [2]Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL

Subnuclear localization of DNA has an important role in regulation of transcription. A number of highly expressed genes are dynamically recruited to the nuclear periphery upon transcriptional activation in yeast. We are using the stress-inducible INO1 gene in yeast as a model system to study the mechanism and functional significance of gene recruitment to the nuclear periphery. Peripheral relocalization of INO1 is controlled by cis acting elements; when INO1 and flanking sequences were integrated at a distant locus in the genome, this locus relocalized to the nuclear periphery upon INO1 activation. Using this system, we have identified cis acting elements that target INO1 to the nuclear periphery. We have identified an 8bp sequence that is necessary and sufficient for gene recruitment. Point mutations in this gene recruitment sequence (GRS) in the INO1 promoter led to a defect in transcriptional activation, suggesting that peripheral localization promotes transcription. Peripheral targeting of either INO1 or the GRS alone was blocked in mutants lacking a number of components of the nuclear pore complex and associated factors. Furthermore, we find a protein activity in yeast lysates that binds with sequence specificity to the GRS. Finally, the GRS was sufficient to target DNA to the nuclear periphery in the distantly related yeast species Schizosaccharomyces pombe, suggesting that this targeting mechanism is conserved over at least one billion years of evolution.

Chloroplasts and Mitochondria (2295 – 2318)

2295/B1

Stress Induces the Assembly of RNA Granules in the Chloroplast of Chlamydomonas Reinhardtii.

J. Uniacke, W. Zerges; Biology, Concordia University, Montreal, QC, Canada

Eukaryotic cells under stress repress the translation of many mRNAs and localize them to cytoplasmic RNA granules, but no such granules have been described in organelles or prokaryotes. Using fluorescence confocal microscopy, we show that specific stress stimuli induce the assembly of RNA granules in an organelle with bacterial ancestry, the chloroplast of *Chlamydomonas reinhardtii*. These chloroplast stress granules (cpSGs) form during oxidative stress, and disassemble during recovery from stress. Like mammalian stress granules, cpSGs contain poly(A)binding protein and the small, but not the large, ribosomal subunit. In addition, mRNAs are in continuous flux between polysomes and cpSGs during stress. Localization of cpSGs within the pyrenoid reveals that this chloroplast compartment functions in this stress response. The large subunit of Rubisco also assembles into cpSGs and is known to bind mRNAs during oxidative stress, raising the possibility that it plays a role in cpSG assembly. This discovery within such an organelle suggests that mRNA localization to granules during stress is a more general phenomenon than currently realized.

2296/B2

Age-Associated Morphology and Metabolism in Mouse and Hamster Oocyte Mitochondria.

F. Castora[1,2], F. Simsek-Duran[1], F. Li[3], K. Wyatt[3], J. Swanson[3], H. W. Jones[4]; [1]Physiological Sciences, Eastern Virginia Medical School, Norfolk, VA, [2]Neurology, Eastern Virginia Medical School, Norfolk, VA, [3]Biology, Old Dominion University, Norfolk, VA, [4]Obstetrics and Gynecology, Jones Institute for Reproductive Medicine, Eastern Virginia Medical School, Norfolk, VA

Mammalian oocytes have striking variance in their pregnancy potential. In humans, about one in five oocyte-sperm meetings results in a viable pregnancy. This variance is due, in part, to the high percentage of aneuploid chromosomes found in the human oocyte at any age. The mechanism by which age-associated aneuploidies are produced is likely dependent upon a rise in oxidative stress, a decrease in ATP, and an increase in reactive oxygen species, all consequences of mitochondrial deficiency. OBJECTIVE: Mice and hamsters were chosen as models for mammalian oocyte biogenesis. Structural and biochemical changes associated with increasing age in the mitochondria of these rodent oocytes were assessed. METHODS: Mitochondria in the ova of young(2 month old)and old (10-14 month old) mice and hamsters were analyzed for normal morphology and number by transmission electron microscopy (TEM). Quantitation of mitochondrial DNA (mtDNA) in individual oocytes was by real-time PCR. Levels of ATP from the oocytes of young and aging mice and hamsters were measured by a chemiluminescent luciferase/luciferin assay. RESULTS: TEM analysis suggested that as the animal ages the mitochondrial morphology undergoes distinct changes. In old hamsters the cristae are dramatically decreased in number and length compared to those in young animals. Individual hamster oocytes from young animals contain, on average, 144 ± 8.5 fmoles of ATP. The level of ATP in old hamsters (115 ± 12.1 fmoles) is diminished approximately 20% compared to ATP in young hamsters. An even greater reduction in ATP levels is seen in aged compared to young mouse oocytes (557 ± 42 vs 414 ± 49 fmoles). An analysis of mtDNA number in hamster oocytes indicates that there is also a decrease in mtDNA molecules per oocyte as the animal ages (11,150 ± 1,324 vs 8,735 ± 2,642). CONCLUSIONS: Mitochondria in mice and hamsters undergo significant morphologic change with aging. These changes affect the structure of the mitochondrial inner membrane and result in a decreased capacity to generate energy necessary for normal oocyte function. In hamsters, the decrease in ATP levels seen in aged oocytes is correlated with a similar decrease in the number of mtDNA molecules.

2297/B3

Ugo1 Is Required for Outer and Inner Mitochondrial Membrane Fusion at a Post-membrane Tethering Step.

S. C. Hoppins[1], J. S. Horner[1], M. J. McCaffery[2], J. M. Nunnari[1]; [1]Molecular and Cellular Biology, University of California, Davis, Davis, CA, [2]Department of Biology and Integrated Imaging Center, Johns Hopkins University, Baltimore, MD

In yeast, there are three proteins that are essential for mitochondrial fusion. Two are the highly conserved dynamin-related GTPases, Fzo1 and Mgm1, that reside in the outer and inner membranes, respectively. At each membrane, Fzo1 and Mgm1 control the distinct steps of

membrane tethering and lipid mixing during membrane fusion. The third essential component is Ugo1, an outer membrane protein in the mitochondrial transport protein family that interacts with Fzo1 and Mgm1. To address the precise role of Ugo1 in fusion, we characterized its structure and function in vitro, where the membrane tethering and lipid mixing steps of both outer and inner membrane fusion events can be resolved. Protease protection analysis shows that Ugo1 contains three transmembrane domains in the amino terminal half of the protein, consistent with the known interactions of Ugo1 with Fzo1 and Mgm1 in the outer and inner membranes respectively. In addition, native gel electrophoresis reveals that Ugo1 exists as a dimer and that this state is critical for Ugo1 function in fusion. To further characterize the role of Ugo1 in fusion, temperature sensitive alleles defective in fusion were created and used in an analysis of mitochondrial fusion in vitro. This functional analysis of Ugo1 indicates that it is required distinctly for both outer and inner membrane fusion after membrane tethering, indicating that it functions specifically at the lipid mixing step of fusion. Analysis of the oligomeric state of these temperature sensitive mutant proteins also reveals that the Ugo1 dimer is destabilized to various degrees in all Ugo1 mutants. Together, these data suggest a model in which dynamic interactions of Ugo1 oligomers with the fusion DRPs mediate the final steps/stages of mitochondrial membrane fusion.

2298/B4

Analyses of Human Mitochondrial DNA and Y Chromosome Haplotypes from Haiti Reveal Regional Variations and Differential Affinities with Various African Populations.

J. Wilson[1,2], A. Samson[2], L. Marcelin[2], B. A. Jackson[1,2]; [1]Biomedical Engineering and Biotechnology Program, University of Massachusetts, Lowell, MA, [2]Biotechnology and Forensic DNA Program, MassBay Community College, Wellesley, MA

The objective of this study was to analyze the mitochondrial DNA (mtDNA) hypervariable segment (HV) Y chromosome single nucleotide polymorphisms (SNPs) from human subjects primarily from rural areas in Haiti to determine population substructuring. These loci are useful due to the apparent lack recombination, predictable linear maternal or paternal inheritance pattern and polymorphic display across world populations. The overall HVS-1 mtDNA sequence diversity values, average number of pairwise differences, percentage of segregating sites, and distributions of pairwise differences based on raggedness index were similar between the northern and southern Haitian subgroups. There appeared to be little mtDNA haplotype sharing between the northern and southern Haitian subgroups. Analysis of MOlecular Variance (AMOVA) revealed that 3.27% of the maternal genetic variance was attributed to difference in geography in Haiti. Given the high diversity of mtDNA haplotypes, differentiation was also accessed at the haplogroup level. When FST pairwise differences were analyzed based on 17 generalized haplogroups found in the Haitian sample, they continued to show significant differences between northern and southern Haiti. 42% of the mtDNA haplotypes in the Haitian data set were unique in comparison to a world-wide database. When matches were found, the highest number were from west central Africa, followed by west Africa, while east Africa were negligible. Also principle components revealed considerable differentiation between west and west central African populations with African Diaspora midway between the two. Using these as parental populations, it was possible to derive relative African intra-continental admixture coefficients for Haitians and other African Diaspora populations. Our results showed that the African maternal genetic component of Haitians had slightly higher west central African admixture than the African Americans and Dominicans, but considerably less than Afro Brazilians. However, regional differences were not as substantial when analyzing Y chromosome lineages.

2299/B5

Mitochondrial Fragmentation Is Required for Mitotic Entry.

S. Lee[4,1], Y. Park[4,1], X. Sun[2], G. Chan[3], H. Cho[4,1]; [1]Biochemistry, Ajou university school of medicine, Suwon, South Korea, [2]Oncology, Cross Cancer Institute, Edmonton, AB, Canada, [3]Oncology, University of Alberta, Edmonton, AB, Canada, [4]Molecular Science and Technology, Ajou university, Suwon, South Korea

Accurate DNA duplication and its even partition into two daughter cells are tightly regulated, however, the mechanisms underlying organelle biogenesis and partitioning in cell cycle remains largely unknown. Mitochondria are highly interconnected organelles that continually fuse and divide. In interphase, highly interconnected mitochondria are observed but no longer exist in mitotic cells, suggesting that the mitochondrial reorganization may occur during G2/M transition. Since mitochondrial fission and fusion processes determine the morphology and interconnectivity of mitochondria, we suppressed the mitochondrial fission by depleting one of fission promoting molecules, hFis1 by shRNA. Cells lacking hFis1 showed a significant elongation of mitochondria and cell cycle progression into mitotic phase was seriously impaired as evidenced by low mitotic index under time lapse microscopy and using aceto-orcein staining. In these cells, the mitotic cyclin B1 failed to accumulate during G2/M transition and phosphorylation of histone H3, a mitotic marker, was not detected, indicating that mitochondrial fragmentation is indispensible for proper mitotic entry. In fact, depletion of both hFis1 and Opa1, a critical component of mitochondrial fusion, led to mitochondrial fragmentation and is capable of restoring the impaired mitotic entry. Together, the data elucidate that mitochondrial dynamics are coupled with cell cycle progression and mitochondrial fragmentation is needed for mitotic entry.

2300/B6

Characterization of IHG-1 - a Novel Mitochondrial Amplifier of TGFβ Signalling.

F. B. Hickey, J. Corcoran, B. Griffin, U. Bhreathnach, F. Furlong, D. Cottell, F. Martin, C. Godson, M. Murphy; Diabetes Research Centre, University College Dublin, Dublin 4, Ireland

Induced in high glucose-1 (IHG-1) is a novel gene that we have described to be upregulated in *in vitro* and *In Vivo* models of fibrotic diseases, and in human diabetic kidney disease. Our evidence indicates that IHG-1 amplifies responses to TGFβ via stabilisation of phospho-smad3. IHG-1 is highly conserved across phyla and a mitochondrial localisation signal (mls) is its only predicted functional domain. We have investigated the significance of the mls in IHG-1. We have confirmed the mitochondrial location of IHG-1 by subcellular fractionation, immunocytochemistry and confocal microscopy following overexpression of V5-tagged IHG-1 in mammalian cells. Additionally, using an anti-IHG-1 polyclonal antibody we have confirmed the mitochondrial localisation of endogenous IHG-1. Further analysis of the submitochondrial localisation of IHG-1 has been achieved through immunogold cryosection electron microscopy. Overexpressed V5-tagged IHG-1 was found to be located predominantly in the inner mitochondrial membrane. Interestingly, IHG-1 expression is upregulated by ROS.

We have investigated the function of IHG-1 in cellular processes controlled by mitochondria, including oxidative stress, apoptosis and mitochondrial fission and fusion. Our preliminary data support a role for IHG-1 in these processes.

2301/B7

Functional Analysis of Mammalian m-AAA Proteases.

S. Ehses[1], A. Bernacchia[2], E. Rugarli[2], T. Langer[1]; [1]Institute for Genetics, University of Cologne, Cologne, Germany, [2]Division of Biochemistry and Genetics, Istituto Nazionale Neurologico "C. Besta", Milan, Italy

The m-AAA protease, an ATP-dependent proteolytic complex in the inner mitochondrial membrane, controls mitochondrial protein quality and, acting as a processing enzyme, regulates mitochondrial biogenesis in yeast. Several isoenzymes of mammalian m-AAA proteases with variable subunit composition exist in mammals. Reconstitution experiments in yeast indicate that all m-AAA isoenzymes exert proteolytic activity. Murine m-AAA proteases are composed of the subunits Afg3l1, Afg3l2 and paraplegin, which are expressed in a tissue-specific manner. Loss-of-function mutations in the m-AAA protease subunit paraplegin cause the neurodegenerative disease hereditary spastic paraplegia (HSP) which is mainly characterized by a cell-specific axonal degeneration. Similarly, Afg3l2 mutant mice exhibit neuropathological features with a severe defect in axonal development. However, the molecular basis of these neuron-specific phenotypes as well as cellular functions of mammalian m-AAA proteases in general remain unclear. To provide further insights into functions of mammalian m-AAA isoenzymes on a cellular level, different subunits were transiently downregulated in MEFs using RNA interference. Our findings revealed crucial functions of m-AAA proteases in the regulation of mitochondrial morphology which may provide new insights into the molecular mechanisms of axonopathies caused by the loss of m-AAA protease subunits in mammals.

2302/B8

Active Akt Localizes to the Intermembrane Space of Mammalian Brain Mitochondria.

S. Walsh, E. A. Jimenez, M. Teitell, C. Koehler; Chemistry and Biochemistry, UCLA, Los Angeles, CA

Recent studies have identified new pathways in the mitochondrial intermembrane space (IMS), including an oxidative folding pathway for the import of proteins and phosphorylation-dependent signaling. We are investigating the kinase Akt (also known as Protein Kinase B), a major serine/threonine kinase regulated by extracellular and intracellular signaling pathways and capable of inducing pro-survival and proliferative effects. A variety of proteins, such as transcription factors, apoptotic machinery, and components of glycogen metabolism, are cytosolic and nuclear substrates of Akt. Previously, it was reported that a fraction of the total cellular Akt localizes to the mitochondria in a neuroblastoma tissue culture cell line. We have shown that, though it is present and phosphorylated in both mouse brain and liver cytosol, Akt only localizes to mitochondria isolated from the brain. Furthermore, the fraction of Akt in mouse brain mitochondria is active and localizes to the IMS. Use of a phospho-specific antibody which recognizes phosphorylated Akt consensus sequences suggests there are Akt substrates in mammalian brain mitochondria. At least one substrate is inside the mitochondrion. We are currently investigating the mechanism of Akt import into the mitochondrion and the identity of the mitochondrial substrates of Akt. This line of research will provide insights into Akt regulation and its cellular effects as well as a greater understanding of signaling pathways in the mitochondrial intermembrane space.

2303/B9

Intracellular Distribution of the Feminization Factor Homolog Fem1B in Human Epithelial Cells.

B. Lelouvier, J. A. Martina, R. Puertollano; Laboratory of Cell Biology, Cell Biology and Physiology Center, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD

The human Fem1 gene family, mammalian homologs of the feminization factor fem-1 of *Caenorhabditis elegans* consists of three members designated FEM1A, FEM1B and FEM1C. These genes encodes for ankyrin repeat proteins, and among them Fem1B seems to be involved in apoptosis. However Fem1B is mostly unstudied and little is known about its expression and function. The distribution and dynamics of transfected Fem1B were therefore examined by confocal microscopy on living retinal pigment epithelia cell ARPE 19. Green fluorescent protein tagged version of Fem1B (GFP-Fem1B) transfected in ARPE19 cell spreads throughout the cytosol and nucleus but is particularly enriched in the mitochondria. Mitochondria of cell transfected with Fem1B have normal shape and distribution and live cell imaging revealed fast mitochondrial movements and numerous fusion/fission events similar to those observed in non transfected cells. Using Fluorescence Recovery After Photobleaching (FRAP) and Fluorescence Loss In Photobleaching (FLIP) we found that Fem1B present a high motility between the mitochondria and the cytosol. Time-lapse confocal analysises were also performed to study the dynamic interaction of mitochondria labeled with GFP-Fem1B and vesicles expressing markers of endosomal and lysosomal compartments. To further study the function of Fem1B we will analyse the consequences of its depletion by siRNA in ARPE19 and Hela cells. This study brings more information about FemIB distribution and should provide new prospects about its function. Considering the major implication of mitochondria in the programmed cell death, the expression of Fem1B in this compartment is consistent with its previously suggested involvement in apoptosis.

2304/B10

Hydrogen Peroxide Treatment Alters Peroxiredoxin 3 Expression in Drosophila Melanogaster Adult Males.

S. Pottinger-Francis[1], P. L. Joseph[2], T. M. Onorato[1]; [1]Natural & Applied Sciences, LaGuardia Community College, Long Island City, NY, [2]Biological Sciences, Kingsborough Community College, Brooklyn, NY

Peroxiredoxin 3 (Prx3) is a mitochondrial thioredoxin-dependent peroxidase that neutralizes excess peroxides, and helps protect against oxidative-stress-induced apoptosis. The gene *DPx-5037* in *Drosophila melanogaster* encodes for a homolog of mammalian Prx3. *DPx-5037* mRNA is expressed in all stages of *Drosophila* development. As with Prx3 in mammalian cells, *Drosophila* Prx3 is cyto-protective; over expression of Prx3 in Schneider S2 cells prevents cell death in response to stressors such as hydrogen peroxide and cadmium. We investigated whether hydrogen peroxide treatment affects Prx3 expression in *Drosophila melanogaster* adult males, since oxidative stress is known to have deleterious effects on male reproductive biology. Adult male flies (day 0-5) were dry starved for 3 hours (h), and exposed to

filter paper soaked in 5% sucrose containing 0% (control), 0.3%, 0.9%, or 1.8% hydrogen peroxide. After 48h, total RNA and protein were extracted from whole flies and used for reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analyses, respectively. All doses of hydrogen peroxide caused an approximate 2-fold increase in Prx3 mRNA levels, with the greatest increase occurring at the 0.3% hydrogen peroxide concentration. Interestingly, the higher doses of hydrogen peroxide caused a dramatic decrease in Prx3 protein levels after 48h compared to the control. However, treatment for 48h with 0.3% hydrogen peroxide did not result in decreased Prx3 protein levels. In summary, these preliminary findings show that 48h exposure to 0.3%, 0.9%, and 1.8% hydrogen peroxide has differential effects on Prx3 mRNA and protein levels in *Drosophila melanogaster* adult males.

2305/B11

The Effects of Di-(2-Ethylhexyl) Phthalate on Peroxiredoxin 3 Expression in Drosophila Melanogaster Adult Males.

C. A. Ruiz[1], P. L. Joseph[2], T. M. Onorato[1]; [1]Natural & Applied Sciences, LaGuardia Community College, Long Island City, NY, [2]Biological Sciences, Kingsborough Community College, Brooklyn, NY

Phthalates are infused with polymers to increase flexibility of plastics. Exposure to phthalates is ubiquitous, e.g., food containers, toys, baby bottles, IV tubing, blood storage bags, PVC flooring, and household dust. Di-(2-ethylhexyl) phthalate (DEHP), one of the most commonly used phthalates, has adverse effects on the male reproductive system. Moreover, DEHP may shorten the lifespan of the fruit fly, *Drosophila melanogaster*, as well as increase lipid peroxidation, an indicator of oxidative stress. Peroxiredoxin 3 (Prx3) is a mitochondrial thioredoxin-dependent peroxidase that neutralizes excess peroxides, and helps protect against oxidative-stress-induced apoptosis. Mitochondrial Prx3 levels are significantly increased in an immortalized mouse spermatocyte-derived germ cell line, GC-2spd(ts), after 24 hour (h) exposure to 200µM mono-(2-ethylhexyl) phthalate, the biologically active metabolite of DEHP. Therefore, this study determined whether DEHP affects Prx3 expression in *Drosophila* adult males. Adult male flies (day 0-5) were dry starved for 3h, and exposed to filter paper soaked in 5% sucrose containing 0 mM (control), 0.5 mM, and 1 mM DEHP. After 48h, total RNA and protein were extracted from whole flies and used for reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot analyses, respectively. Exposure to DEHP (0.5mM and 1mM; 48h) did not alter Prx3 mRNA expression. However, 48h treatment with 0.5 mM and 1mM DEHP resulted in a 1.5-fold increase in Prx3 protein levels. In summary, these preliminary findings show that 48h exposure to 0.5 mM and 1 mM DEHP has no affect on Prx3 mRNA levels, but causes an increase in Prx3 protein levels.

2306/B12

Mitochondrial Morphology: Quantifying Topological Network Properties in 3 Dimensions.

S. Rafelski[1], M. P. Viana[2], K. S. Thorn[1], L. F. Costa[2], W. Marshall[1]; [1]Biochemistry & Biophysics, UCSF, San Francisco, CA, [2]Inst. de Fisica de Sao Carlos, Universidade de Sao Paulo, Sao Carlos, Brazil

A fundamental unsolved question in the post-genomic era is how the 3-dimensional geometry of cellular structures is generated by networks of molecular interactions. Mitochondria are an ideal system in which to approach this question. Mitochondrial morphology ranges from small individual organelles to giant reticular networks and affects mitochondrial functions. In budding yeast mitochondria compose a dynamic tubular network at the cell periphery, which undergoes subtle, consistent changes in morphology under different physiological conditions. We aim to understand the mechanisms that regulate the normal range of reticular structures. We have developed a method to quantify the 3D interconnected morphology of the mitochondrial network by considering the mitochondria as a network of edges (the tubules) and nodes (the branch points where tubules connect). Live mitochondria are rapidly imaged in 3D at high resolution using Spinning-disk Confocal microscopy. Specialized segmentation and 3D skeletonization methods are applied to the image stacks to extract the 3D mathematical graph of the mitochondrial network. For validation we create theoretical microscope images from pre-determined skeletonized structures and compare these to the 3D skeletons obtained by our "mito-graph" methodology. These tests are used to optimize the accuracy of the 3D skeletons and determine their limits, imposed by microscopy imaging. We then apply concepts and methods from complex networks research to characterize the connectivity and clustering of the tubules. This quantification technique provides us with measurements of the arc lengths, density, and connectivity of mitochondrial tubules in the cell and how the connections change as individual tubules undergo constant fusion and fission dynamics or individual cells progress through the cell cycle. In our first analysis we focus on how the properties of the network change with tubule density at the surface. We compare measurements of real mitochondrial networks with simulated networks created by joining points (nodes) on the surface of a sphere with edges following a set of rules to explore the minimum requirements necessary to generate quantitatively realistic mitochondrial networks.

2307/B13

Iba57p Is a Mitochondrial Fe/S Cluster Biogenesis Factor with Substrate Specificity.

C. L. Gelling[1], U. Mühlenhoff[2], N. Richhardt[2], R. Lill[2], I. W. Dawes[1]; [1]School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, NSW, Australia, [2]Institut für Zytobiologie und Zytopathologie, Philipps-Universität, Marburg, Germany

Iron-sulfur (Fe/S) clusters serve as prosthetic groups for a large and diverse set of proteins but are initially synthesized onto a small number of common scaffold proteins. Most work on eukaryote Fe/S biogenesis has focused on such generalized factors of the core biosynthetic machinery but biogenesis factors specific for certain apo-protein targets may also play an important role. In order to identify Fe/S cluster biogenesis factors in the target specific Isa1/2p pathway, a screen of the genome-wide *Saccharomyces cerevisiae* deletion strain collection was performed. Iba57p, a previously uncharacterized mitochondrial protein similar to tetrahydrofolate-binding enzymes, was identified in this screen and was found to stably interact with Isa1p and Isa2p. The *iba57* deletion mutant strain displayed a number of Fe/S cluster-related phenotypes including loss of the mitochondrial genome. Repression of Iba57p resulted in defective Fe/S cluster assembly onto mitochondrial, but not cytosolic, proteins of the aconitase family. Interestingly, the function of mitochondrial radical-SAM-Fe/S proteins was also impaired by repression of Iba57p, even though iron incorporation into the same proteins was not, indicating some specific involvement of Iba57p in the self-destructive catalytic cycle of such enzymes. Homologues of Iba57p have been identified in most eukaryotic organisms, including humans, and expression of the human homologue was able to complement the growth defects of the *S. cerevisiae* mutant, indicating that the molecular function of proteins in this family is conserved.

2308/B14

Real-Time Imaging of Mitochondria in Transgenic Zebrafish Expressing Mitochondrially Targeted GFP.

M. Kim[2,3], K. Kang[1], S. Choi[1,3]; [1]Biomedical Sciences, Chonnam National University, Gwangju, South Korea, [2]Biological Sciences, Sookmyung Women's University, Seoul, South Korea, [3]NICHD, National Institutes of Health, New York, NY

Mitochondria maintain a web-shaped network in cells through a balance between fusion and fission. Under certain physiological and pathological conditions, this balance is breached and as a result, change in mitochondrial morphology ensues. Real-time monitoring of such change is of significant importance for studying mitochondrial physiology and pathology, such as apoptosis, aging and neurodegeneration. Numerous studies have been conducted in animal cell culture systems concerning mitochondrial morphology change. However, very little is known to date about the real-time changes in mitochondrial morphology at the organism level due to difficulties in observation and administration of mitochondria disrupting drugs. Here we report the generation of transgenic zebrafish (Danio rerio) expressing mitochondrially targeted green fluorescent protein (GFP). The transparency of transgenic zebrafish embryos made it possible to monitor mitochondrial morphology in real time and in vivo. Since zebrafish inhabit fresh water, incubating zebrafish in drug-dissolved water sufficed to administer drugs to the zebrafish. We observed real time and in vivo fragmentation of mitochondria in the transgenic embryos upon incubation in water with the following apoptosis-inducing drugs: valinomycin, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP) and staurosporine. Thus the transgenic zebrafish we generated could provide a platform for research on apoptosis and mitochondrial physiology, and a screen for apoptosis modulating drugs and facilitate study of the pathogenesis of apoptosis-related diseases.

2309/B15

Characterization of Sequence Motifs in F1-Atpase Gamma Subunit Presequence.

S. Lee, D. Lee, I. Hwang; Life Science, POSTECH, Pohang, South Korea

Mitochondria have their own DNA, but most of mitochondrial proteins are coded in genomic DNA in the nucleus, synthesized in cytosol and transported into mitochondria via outer and inner membrane translocator complexes. Presequence, the signal peptide located in the N-terminus of nuclear encoded mitochondrial proteins is sufficient for protein import from cytosol into mitochondria. However, there is little sequence homology among presequences, also the general features of presequences are not fully understood. In this study, we identified functional sequence motifs of F1-ATPase gamma subunit's presequence by examining the mitochondrial targeting efficiency of various mutant presequences fused with green fluorescence protein (GFP) reporter proteins. We generated several reporter proteins having deletion or alanine substitution mutant presequences and analyzed using in vivo import system in Arabidopsis thaliana leaf protoplasts. The data showed that the motifs DQEEG and VVRNR played a major role in translocation into mitochondria. When these two motifs were substituted to alanine, the targeting to mitochondria was completely blocked, but the point substitution of just a few amino acids in these motifs were not affected to targeting efficiency. We also revealed that some of motifs have a redundancy or synergistic effect with the other motif block, although most of pairs which contain motif candidates have show the similar efficiency with single substitution mutants. This study demonstrates that the presequence contains multiple sequence motifs, and some of them have a critical function in mitochondrial protein translocation.

2310/B16

Role of Mitochondria in Inflammation-Associated Neurogenesis Impairment.

L. A. Voloboueva, J. Emery, R. G. Giffard; Anesthesia, Stanford University, Palo Alto, CA

The purpose of this study was to investigate the role of mitochondria in neuronal differentiation, and the effects of mitochondria protection on inflammation-induced impairment of neurogenesis. The birth of new neurons within certain parts of central nervous system continues throughout life, and the neurogenesis process has been extensively studied in the last decade. Neural stem cells are widespread within the adult brain but are instructed to differentiate into neurons only in discrete brain areas with specific local environment. However, new neurons are also produced in brain following injuries such as targeted ablation of cortical neurons, gross trauma, and stroke. Some newly formed neurons integrate functionally into the neuronal circuitry. It has been demonstrated that only a small portion of the newly born neurons survive. Multiple studies have shown that the extensive microglial inflammation and release of pro-inflammatory cytokines characteristic of brain injury strongly suppress neurogenesis. However, the cellular mechanisms of the inflammation-associated neurogenesis suppression remain poorly studied. Cellular mitochondria regulate apoptosis induced by pro-inflammatory cytokines, and are important for cellular differentiation in various cell types. In our studies we used neural precursor cells isolated from neuborn mouse brains. The cells were cultured as neurospheres in the growth culture medium, and subsequently induced to differentiate by plating dissociated neurospheres in the differentiation medium. We observe that inhibition of mitochondrial function suppresses neuronal differentiation and promotes selective death of neuronal progenitor cells in cultured brain cells. The results of our studies also suggest that protection of cellular mitochondria with lactate and thiamine, mitochondrial metabolites, or lipoic acid, a mitochondrial antioxidant, ameliorates the inflammation-associated neuronal progenitor cell death in vitro. Developing strategies to increase survival of new cells produced by "reactive neurogenesis" may contribute to post-injury recovery.

2311/B17

Mitochondrial Morphology Is an Active Factor Regulating Mitochondrial Function.

T. Yu, Y. Yoon; Department of Anesthesiology, University of Rochester, Rochester, NY

Mitochondrial shape change is mediated by fission and fusion of mitochondrial membranes. Although physiological roles of the mitochondrial shape change is not fully understood, it has been suggested that different shapes of mitochondria may reflect particular states of mitochondrial activities. To understand the correlation between mitochondrial morphology and function, we blocked mitochondrial fission in cells and examined its morphological and functional consequences. The dominant-negative mitochondrial fission mutant DLP1-K38A was used to inhibit mitochondrial fission. Cells overexpressing DLP1-K38A showed elongated and entangled mitochondria. Morphological quantification indicated that mitochondrial fission decreased by 2-3 folds, consistent with the generation of overly elongated mitochondria in these cells. Interestingly, however, the mitochondrial fusion frequency also reduced to a similar extent when mitochondrial fission was

inhibited, suggesting an inter-dependence of fission and fusion processes. We found that inhibition of mitochondrial fission slowed down cell growth and proliferation, suggesting an overall decrease in cellular functions. In experiments testing the mitochondrial activity, we found that cells overexpressing DLP1-K38A have a significantly higher mitochondrial membrane potential compared to control cells. On the other hand, these cells showed decreased oxygen consumption. Further experiments measuring the mitochondrial membrane potential in the presence of the mitochondrial ATP synthase inhibitor oligomycine indicated that the proton pumping activity of the electron transport chain in these cells decreased below the normal activity. These cells also exhibited a lower reactive oxygen species level than control cells. These results demonstrate that blocking mitochondrial fission causes a decreased activity of the mitochondrial electron transport chain and a potential defect in dissipating the electrochemical gradient across the inner mitochondrial membrane. Our results suggest that changing mitochondrial morphology alters mitochondrial function, supporting the notion that mitochondrial shape change is an active factor participating in controlling mitochondrial activity.

2312/B18

A Quantitative Mitochondrial Fusion Assay via Split Renilla Luciferase Complementation.

H. Huang[1], S. Choi[1,2], M. A. Frohman[1]; [1]Department of Pharmacology, Stony Brook University, Stony Brook, NY, [2]Department of Biomedical Sciences, Chonnam Medical School, Hak-Dong, South Korea

Mitochondria maintain physiological functions by keeping the balance of fusion and fission. The mechanistic studies of mitochondrial fusion rely heavily on the mitochondrial fusion assay established several years ago. However, the classic way to measure mitochondrial fusion is labor-intensive and not easily quantitative. To solve this problem, we developed a quantitative mitochondrial fusion assay by taking advantage of the split synthetic Renilla luciferase (hRluc) complementation system. We fused split fragments of hRluc with a mitochondrial matrix-targeting sequence and introduced each fragment into HeLa cells to make stable expression, which enables us to monitor the mitochondrial fusion extent via chemiluminescence. The reconstitution of split hRluc can be detected within half an hour and peaks at around 3 hours, representing the balance between mitochondrial fusion and gradual degradation of the recombinant proteins. To further validate this assay, we tested our cells on several mitochondrial inhibitors, which have been shown to inhibit mitochondrial fusion. Our results also showed significant decreases in luciferase activities under drug treatments. It has also long been acknowledged that mitofusin1/2 are the pivotal mediators of mitochondrial fusion, absence of which certainly lead to mitochondrial fusion dysfunction. We have performed the quantitative assay in knock-out cells of mitofusions and found almost no signal for reconstituted luciferase in fused cells. Taken together, we have developed a quantitative and less labor intensive mitochondrial fusion assay, which can potentially solve questions that can not be addressed by the classis fusion assay.

2313/B19

Identification and Characterization of Fungal Retrograde Signaling Genes.

E. S. Unlu, D. M. Gordon; Biological Sciences, Mississippi State University, Mississippi State, MS

Changes in gene expression in response to mitochondrial dysfunction is mediated by components of the retrograde signaling pathway. This molecular link between mitochondria and the nuclear genome is controlled by three key signaling proteins: Rtg1p, Rtg2p, and Rtg3p. Work by several labs has shown that defects in mitochondrial function is first sensed by cytosolic Rtg2p which transmits the signal to the Rtg1p/Rtg3p transcription factor triggering its translocation from the cytosol to the nucleus. This results in the upregulation of genes encoding metabolic enzymes and stress response proteins to compensate for loss of mitochondrial activity. Retrograde signaling genes have been well characterized in the budding yeast *Saccharomyces cerevisiae* but very little is know about the retrograde response of other fungi. To identify retrograde signaling proteins in other yeast, the protein sequence encoded by the *Saccharomyces cerevisiae RTG2* gene was used to search for fungal homologs using NCBI BlastP followed by additional analysis using the T-Coffee Multiple Sequence Alignment program. We selected four species having uncharacterized ORFs with more than 66% amino acid identity to Rtg2p for further analysis: *Ashbya gossypii*, *Candida glabrata*, *Vanderwaltozyma polyspora*, and *Kluyveromyces lactis*. In *S. cerevisiae*, cells deleted for *RTG2* lose aconitase protein and are unable to grow on synthetic media lacking proline. To determine whether the putative *RTG2* genes we identified encode bonefide regulators of the retrograde response pathway, we will test their ability to complement the defects associated with the *rtg2* mutant using our *RTG2* shuffle strain. By characterizing the molecular players involved in fungal retrograde signaling we hope to understand the evolution of the cellular mechanisms induced to compensate for loss of mitochondrial activity in fungi.

2314/B20

A Novel Allele of Mgm1 Retains Mitochondrial Genomes and Fusion Capabilities Despite a Loss of Respiratory Function.

R. M. DeVay[1], J. McCaffery[2], J. Nunnari[1]; [1]Molecular and Cellular Biology, University of California, Davis, Davis, CA, [2]Biology and Integrated Imaging Center, Johns Hopkins University, Baltimore, MD

Mitochondrial fusion is a highly conserved and functionally important process that evolved to create a more connected compartment that facilitates content exchange and access to mitochondrial DNA (mtDNA). In yeast, there are three essential fusion proteins. Two are the highly conserved dynamin-related GTPases (DRPs), Fzo1 and Mgm1, residing in the outer and inner membranes, respectively. At each membrane, these DRPs are required for the distinct steps of membrane tethering and lipid mixing in mitochondrial fusion. The third component is Ugo1, an outer membrane protein in the mitochondrial transport protein family that interacts with Fzo1 and Mgm1. Yeast cells that harbor deletions of the genes encoding these essential fusion proteins have fragmented mitochondria and completely lose their mitochondrial DNA (mtDNA) and consequently cannot respire and grow on non-fermentable carbon sources. In addition to its role in fusion, Mgm1, the inner membrane DRP, has been implicated in the maintenance of cristae structures. We have identified a unique Mgm1 allele that supports mitochondrial fusion and maintains mtDNA in cells, but surprisingly fails to support growth on non-fermentable carbon sources. Characterization of this mutant reveals that its mtDNA is intact and transcribed, but mitochondrial transcripts are not translated, which is the likely cause of its inability to respire. Additionally, electron microscopy analysis reveals that mutant cells do not possess normal cristae structures. Thus, our data indicate that Mgm1 plays multiple roles in the maintenance of mitochondrial function and suggest that its roles in cristae maintenance and fusion are separable. Our findings further suggest that Mgm1 function is not only required to maintain cristae, but

also to organize the inner membrane so that it is competent for mitochondrial protein translation, a process intimately linked to the inner membrane.

2315/B21

Targeted Selection for Mitochondrial DNA Mutations in Drosophila.

H. Xu, P. O'Farrell; Biochemistry & Biophysics, UCSF, San Francisco, CA

Mitochondrial DNA mutations give rise to human disease, and accumulation of mtDNA damage is thought to contribute to age-related disorders. Animal models are much needed to investigate the occurrence, pathogenicity and transmission-pattern of mitochondrial mutations. The random of segregation of the multiple-copied of mtDNA in each cell results in a shifting representation of different mitochondrial genotypes in the cells of individuals, which greatly impeded the application of common functional genetic tools to study mtDNA. There is no technique available for targeting mutagenesis of mtDNA or conditionally selecting for altered genomes in any metazoans. Generation of experimental animals carrying inheritable mtDNA mutations remains challenge. We have developed a method to efficiently select mtDNA mutations by expression of mitochondrially-targeted restriction enzymes, such as XhoI (mitoXhoI). MitoXhoI expression in the germline led to sterility, but occasional females gave escaper progeny carrying homoplasmic mtDNA mutations eliminating the single XhoI restriction site of mtDNA. One of the substitution mutations affecting cytochrome C oxidase, mt:Co1R301L, is male sterile, but otherwise healthy. Another mutation, mt:co1R301S, exhibits a wide range of defects, including growth retardation, retinal degeneration, muscular disorganization, male sterility, and reduced life span. Some of these defects resemble features of human mtDNA diseases. Successful use of other restriction enzymes suggests that additional nuclease specificities will allow selection of diverse mutations, which can be used to model human mtDNA diseases. Selective expression of mitochondrially targeted enzymes in the germline should similarly allow selection of mitochondrial mutations in other metazoans.

2316/B22

Pathways to Divide Mitochondria in C. Elegans.

B. Head, A. Van Der Bliek; Biological Chemistry, UCLA School of Medicine, Los Angeles, CA

Identification of the fundamental players in mitochondrial division -- Drp-1, Fis-1, and Mdv-1 -- has been useful in extending our basic knowledge concerning this essential process. However, it is becoming apparent that many other proteins and regulatory steps contribute functionally to mitochondrial division. Using the model organism C. elegans, we analyzed the drp-1 gene by reverse genetics. Chromosomal deletions in drp-1, demonstrate that DRP-1 is not essential for all mitochondrial divisions and, incredibly, not essential for life. We, therefore, sought out additional proteins and/or pathways that contribute to mitochondrial division. Candidate gene screening led to the identification of a putative protein complex that affects mitochondrial division in the absence of DRP-1. The putative complex consists of a CHCHD protein (M176.3), the mitofilin proteins, and a novel protein we have named SWD-1. SWD-1 localizes to the mitochondrial outer membrane as shown by conventional fluorescence tagging and protease protection experiments. Endogenous SWD-1 also localizes to mitochondria. Loss of SWD-1 causes mitochondrial distortions similar to those observed with the loss of M176.3 and the mitofilin proteins. Additionally, we find similar genetic interactions using knockdown of SWD-1, M176.3, or the mitofilins and knockdown of the SAM protein import complex. Mitofilin has been shown to physically interact with the SAM protein import complex hinting at a role in mitochondrial protein import. Additionally, mitofilin has been suggested to directly affect mitochondrial cristae structure. Our data are consistent with either of these functions and demonstrate that mitofilin acts in a complex with M176.3 and SWD-1. This complex of proteins contributes to mitochondrial division in the absence of DRP-1.

2317/B23

An Analysis of Hfis1, a Component of the Mitochondrial Division Apparatus.

A. E. Frazier[1], D. Laine[1], D. Stojanovski[1], J. Gulbis[2], M. Ryan[1]; [1]Department of Biochemistry, La Trobe University, Melbourne, VIC, Australia, [2]Structural Biology Division, The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC, Australia

Mitochondria are typically found in extended reticular networks that spread throughout the cytoplasm. However, many variations in their shape and distribution have been found, depending on the type of cell, tissue and organism. A number of proteins have been linked to the regulation of these networks, often altering them in response to the developmental, energetic and/or signalling status of the cell. Likewise, changes in mitochondrial morphology have been linked to several human diseases, emphasizing the importance of mitochondrial networks to cellular functioning. Our work has concentrated on the study of the mitochondrial outer membrane protein hFis1, a component of the mitochondrial fission machinery. This integral membrane protein is proposed to act in conjunction with the dynamin-related GTPase Drp1, which gets recruited from the cytosol to form the machinery responsible for dividing mitochondrial tubules. Utilizing our crystal structure of hFis1, we have generated several hFis1 mutants to further explore its function during mitochondrial division. Two of these mutants were designed to disrupt a predicted protein interaction pocket in hFis1. By analysing hFis1 expression in cultured mammalian cells utilising a photoactivatable fluorescent protein, we have observed distinctive alterations in the ability of these mutants to induce mitochondrial fragmentation compared to wild type hFis1. Blue-native PAGE analysis indicates that one of these mutants is unable to form native complexes. Together, these results further our understanding of the mechanism by which hFis1 functions in mitochondrial fission.

2318/B24

Eyeless Mutants of Chlamydomonas Reinhardtii Provide Insight into the Assembly of a Complex Photosensory Organelle.

T. Mittelmeier, J. Boyd, C. Dieckmann; Biochemistry and Molecular Biophysics, University of Arizona, Tucson, AZ

The eyespot of the unicellular, photosynthetic alga *Chlamydomonas reinhardtii* is a photosensory organelle required for phototaxis. The eyespot, viewed in the light microscope as an orange-red spot at the equator of the cell, comprises rhodopsin-family photoreceptors in the plasma membrane directly overlying chloroplast envelope membranes and closely apposed layers of carotenoid pigment granules and thylakoid membrane. What factors govern assembly of the eyespot layers? Is localization of the photoreceptors dependent on assembly of the pigment granule layers, or vice versa? *Chlamydomonas* eyeless *eye2* and *eye3* mutant strains have no eyespot visible by light microscopy,

and are unable to phototax.[1] Immunofluorescence microscopy using an antibody against one of the major eyespot photoreceptors[2] showed that the photoreceptor remains correctly localized to a single side of the cell in the eyeless mutants, but is often found in a "stripe" or in several spots, rather than the single large spot observed in wild-type cells. These data indicate that assembly of the pigment granule layers is not required for gross localization of the photoreceptor, but perhaps for formation or maintenance of a cohesive spot. EYE2, found in the eyespot,[3] is a thioredoxin superfamily protein with a potential chloroplast targeting sequence, and may have a chaperone-like function in assembly of the pigment granule layers.[4] The EYE3 protein has homology to a specific family of serine-threonine kinases found in both the eyespot[3] and in the plastoglobules of plant chloroplasts,[5] suggestive of a role for kinase activity in pigment granule synthesis or organization. Polyclonal antiserum raised against EYE3 stains the eyespot pigment granules and is allowing the simultaneous analysis of photoreceptor, cytoskeleton and granule localization in wild-type and other eyespot mutant strains. [1]Lamb et al., 1999, Genetics 153:721-9. [2]Berthold et al., 2008, Plant Cell 6:1655-77. [3]Schmidt et al., 2006, Plant Cell 18:1908-30. [4]Roberts et al., 2001, Genetics 158:1037-1049. [5]Ytterberg et al., 2006, Plant Physiol. 3:984-7.

Autophagy (2319 – 2327)

2319/B25

Structural Basis for Sorting Mechanism of P62 in Selective Autophagy.

Y. Ichimura[1], Y. Sou[1,2], T. Ueno[1], K. Eiki[1], K. Tanaka[2], M. Komatsu[1,2,3]; [1]Department of Biochemistry, Juntendo University School of Medicine, Tokyo, Japan, [2]Laboratory of Frontier Science, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan, [3]PRESTO, Japan Science and Technology Corporation, Kawaguchi, Japan

Impairment of autophagy causes accumulation of ubiquitin- and p62-positive inclusions. Recently, we have reported that constitutive autophagy selectively degrades p62 thereby regulating the intracellular levels of p62. In addition, previous studies revealed that p62 directly interacts with LC3, autophagosomal membrane protein. However, the molecular mechanism of p62 sorting into autophagosomes is not well understood. In this present study, we identified a motif of p62 comprised of 11 amino acids (Ser-334-Ser-344) containing conserved acidic and hydrophobic residues across species, as an LC3 recognition sequence (LRS). The crystal structure of the LC3-LRS complex at 1.56 Å resolution revealed interaction of Trp-340 and Leu-343 of p62 with different hydrophobic pockets on the ubiquitin-fold of LC3. In vivo analyses demonstrated that p62 mutants lacking LC3-binding ability accumulated without entrapping into autophagosomes in the cytoplasm and subsequently formed ubiquitin-positive inclusion bodies as in autophagy-deficient cells. These results demonstrate that intracellular level of p62 is tightly regulated by autophagy through the direct interaction of LC3 with p62 and reveal that selective turnover of p62 via autophagy controls inclusion body formation.

2320/B26

Selective Targeting of Misfolded Proteins in the Endoplasmic Reticulum for Degradation by Autophagy.

P. S. Satpute-Krishnan, R. S. Hegde, J. Lippincott-Schwartz; NICHD, NIH, Bethesda, MD

Accumulation of misfolded proteins is associated with a variety of diseases including neurodegeneration, liver disease, and cystic fibrosis. Misfolded proteins in the endoplasmic reticulum (ER) are typically degraded by ER associated degradation (ERAD), which involves retrotranslocation of the protein to the cytosol and degradation by the proteasome. However, some misfolded proteins are poorly recognized by the ERAD system, necessitating their disposal by other pathway(s) that are not well characterized. To understand these pathways, we created a fluorescent protein (FP)-tagged variant of the prion protein (PrP) that can neither fold nor undergo ERAD. Using time-lapse live cell imaging in combination with other FP-tagged markers, we have analyzed the fate of this misfolding PrP variant. Our findings reveal that misfolded PrP is segregated from other ER-resident proteins and routed into vesicular membrane-bound compartments, some of which are coated with the autophagy marker LC3. These PrP-containing LC3-positive structures are preferentially enriched upon inhibition of lysosomal degradation, suggesting an autophagy-based degradation system. These findings establish a simple and robust system to study selective autophagy of misfolded ER-retained proteins. We are currently using this model to identify the key steps and molecular mechanisms involved in the selective recognition, segregation, and transport of misfolded proteins from the ER to the autophagosome. The findings from this study may provide novel insights into the regulation of degradation pathways utilized by other disease-related, ER-retained proteins.

2321/B27

Molecular Characteristics of the Autophagosome.

T. Melia[1], A. Yamamoto[2]; [1]Cell Biology, Yale University, New Haven, CT, [2]Neurology, Columbia University, New York, NY

Macroautophagy is a stress-response pathway in which cytosol is sequestered in newly formed organelles (the autophagosomes) before being trafficked to the lysosome for degradation. While many of the proteins involved in formation of the autophagosome have been known for many years, virtually nothing is known about the protein complement comprising the mature autophagosome or of the proteins that play a role in the transitions from isolation membrane to autophagosome to autolysosome. Here we isolate autophagosomes from a variety of cell and tissue sources in order to identify common factors associated with the autophagosomal membrane. The overall autophagic character of our membrane isolates is confirmed by density flotation, MAP1LC3-labeling and multi-lamellar morphology (as assessed by cryo-electron microscopy). Membrane-associated proteins were identified by mass-spectrometry. We have screened hits by siRNA knockdown against a variety of autophagy-related assays, including long-lived protein degradation and MAP1LC3 membrane-association, to establish the role of the autophagosomal membrane-associated proteins in both starvation and protein-accumulation responses. This work has culminated in the identification and characterization of novel factors acting upon autophagosome maturation.

2322/B28

Golgi-Resident Small Gtpase Rab33 Interacts with Atg16L1 and Modulates Autophagosome Formation.

T. Itoh[1], N. Fujita[2], K. Ishibashi[1], T. Yoshimori[2], M. Fukuda[1]; [1]Developmental Biology and Neurosciences, Tohoku University, Sendai, Japan, [2]Cellular Regulation, Research Institute for Microbial Diseases, Osaka University, Suita, Japan

Macroautophagy is a mechanism of degradation of cytoplasmic components in all eukaryotic cells. In the process of macroautophagy cytoplasmic components are wrapped by double-membrane structures called autophagosomes, whose formation involves unique membrane dynamics, i.e., de novo formation of a double-membrane sac called the isolation membrane and its elongation. However, the precise regulatory mechanism of isolation membrane formation and elongation remains unknown. In this study we showed that Atg16L1, an essential factor in isolation membrane formation, specifically interacts with Golgi-resident small GTPases Rab33A and Rab33B in their GTP-bound state. Since Rab33 did not interfere formation of a complex including Atg12, Atg5, and Atg16L1 (Atg12-5/16L1 complex), the Atg12-5/16L1 complex is likely to function as an effector of Rab33. Expression of a GTPase-deficient mutant Rab33B (Rab33B-Q92L) induced the lipidation of LC3, which is an essential process in autophagosome formation and depends on the Atg12-5/16L1 complex, even under nutrient-rich conditions, and attenuated macroautophagy, as judged by the degradation of p62/SQSTM1 (sequestosome 1). In addition, overexpression of the Rab33-binding domain of Atg16L1, which was mapped to the latter part of the coiled-coil domain of Atg16L1, suppressed autophagosome formation. Our findings suggest that Rab33 modulates autophagosome formation through interaction with Atg16L1.

2323/B29

Autophagy as a Mechanism Enabling Coxsackievirus Replication.

D. A. Hartley; Basic Sciences, Touro University Nevada, Henderson, NV

Infection of cells with poliovirus and some human rhinovirus serotypes has been shown to induce the formation of autophagic vesicles, presumably to act as a scaffold for replication. Normally autophagy is a process whereby cellular proteins or organelles are digested and their components recycled. Our initial hypothesis led us to determine if autophagy was also used by other picornaviruses, such as group B Coxsackieviruses. Infection of cells with group B serotype 3 Coxsackievirus (CVB3) is able to induce autophagy as monitored by microscopy (punctuate pattern of endogenous LC3B), and western immunoblotting (gel shift of LC3B due to lipidation). Data show co-localization of viral proteins with autophagic vesicles in infected cells. The autophagy induced by CVB3 proceeds with different kinetics than other inducers of autophagy, such as rapamycin. These results suggest that virus induced autophagic vesicles mature at a slower rate, or that the normal processes of vesicle formation are mechanistically faster than maturation leading to a bottle-neck.

2324/B30

In Vivo Reconstitution of Autophagy in Saccharomyces Cerevisiae.

Y. Cao[1,2], H. Cheong[1,2], H. Song[1,2], D. Klionsky[1,2,3]; [1]Life Sciences Institute, University of Michigan, Ann Arbor, MI, [2]Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI, [3]Department of Biological Chemistry, University of Michigan, Ann Arbor, MI

Autophagy is the major intracellular pathway for the degradation and recycling of long-lived proteins and organelles, and it is also involved in both the pathogenesis and prevention of many human diseases. Much progress has been made on the identification and characterization of AuTophaGy-related (ATG) genes, in yeast and in mammals. However, our understanding of the molecular mechanisms of autophagy remains quite limited, and is far from enough to enable us to harness autophagy for therapeutic applications. To better understand the molecular mechanisms, we took a unique and novel approach to study autophagy in yeast. We generated a multiple knockout Saccharomyces cerevisiae strain with 24 ATG genes deleted, and used it for in vivo reconstitution of the autophagy pathway. We determined the minimum requirements for cargo packaging, initial protein assembly at the phagophore assembly site, and conjugation of the ubiquitin-like proteins. Our data also provided us with new insights into autophagy, different from those obtained from in vitro analyses.

2325/B31

Testing a Role for Diacylglycerol Production in the Regulation of Autophagy.

S. Shahnazari[2,1], J. Shiu[1], C. Birmingham[2,1], J. Brumell[1,2,3]; [1]Cell Biology, Hospital for Sick Children, Toronto, ON, Canada, [2]Molecular Genetics, University of Toronto, Toronto, ON, Canada, [3]Institute of Medical Science, University of Toronto, Toronto, ON, Canada

Autophagy is the process by which long-lived proteins and organelles residing in the cytosol are recycled. It can be triggered by starvation and stress but is also thought to play a protective role against the progression of some human diseases including cancer, muscular disorders and neurodegenerative diseases. More recently it has been found that intracellular infection by some bacterial pathogens can also illicit a protective autophagic response by the infected cell. Previously, our lab has shown that a population of Salmonella enterica serovar Typhimurium is targeted by the autophagy system during infection, restricting the growth of these bacteria inside host cells. Autophagic targeting was found to peak 60' post-infection (pi) and was visualized through co-localization of LC3 (a marker for autophagy) with the Salmonella-containing vacuole (SCV). The signal that acts to induce autophagy at a specific site is currently unknown. While previous studies have implicated phospholipids in starvation-induced autophagy regulation, it is not clear which lipids are important nor where and when they act within the cell. Here we use autophagy of Salmonella as a model of autophagy and using a specific probe for diacylglycerol (DAG) show that a population of LC3 positive SCVs also co-localize with DAG. Maximal co-localization of DAG with bacteria precedes peak LC3 recruitment suggesting a causal role. Inhibition of autophagy was found to have no effect on the localization of DAG to the SCV, indicating that DAG generation occurs before the induction of autophagy. Inhibition of DAG biosynthesis results in a 25-fold reduction in LC3 localization to the SCV at 60' pi. DAG was also found to be present on starvation-induced autophagosomes indicated a general signaling role for DAG in autophagy. Furthermore, we have determined using pharmacological inhibitors and siRNA that DAG acts to recruit Protein Kinase C (PKC) isoforms which are involved in autophagy of Salmonella. These studies suggest that DAG is an early signal for the initiation of autophagy.

2326/B32

A Genome-Wide Sirna-Based Screen to Discover Novel Modulators of Mammalian Autophagy.

N. C. McKnight, S. Tooze; Secretory Pathways Lab, London Research Institute, London, United Kingdom

Autophagy is a catabolic mechanism by which cytoplasmic components are sequestered and transported by a double-membraned vesicle called an autophagosome to the lysosome for degradation. This recycling of organelles and macromolecules provides the cell with amino acids in times of nutrient deprivation though we do not know how the process is triggered or regulated. Multiple stresses and misbalances of cellular chemistry can also trigger autophagy. It is a highly regulated process in mammalian cells and its misregulation has been shown to contribute to multiple diseases. In order to find new regulators of mammalian autophagy, we performed a genome-wide screen using the Dharmacon human siRNA library in a stable human cell line expressing GFP-LC3, a specific marker protein for autophagosomal membranes (Chan et al., JBC 282: 25464, 2007). First we incubated the cells with the pooled siRNA oligonucleotide duplexes using a reverse transfection protocol. Then we induced a starvation response by culturing the cells in a buffer devoid of amino acids. This initiated the formation of GFP-LC3-labelled autophagosomes that became visible 'spots.' Using the Cellomics VTi Scan microscope and accompanying quantitative software we measured the effect of specific siRNA-mediated knock-down on multiple parameters including spot count. The assay was performed in 96-well plates and each siRNA pool was tested in triplicate. Including sixteen wells per plate for controls, the 21,000-plus siRNA library was screened in 801 plates. The plates were processed together over a four day period. After accounting for cell death and normalizing the data to the plate means we generated a B-score for each siRNA pool. We then ranked the hits accordingly. We retested the top 500 and bottom 500 candidates (increasing or decreasing autophagosome formation, respectively) following the protocol described above. After deconvolution of the siRNA pools, alternate autophagic assays will be employed on the candidates. Preliminary results from this screen suggest a number of novel candidates have been identified that we will follow and further characterize to elucidate the proteins's roles in autophagy.

2327/B33

A Role for Phospholipase D in the Regulation of Macroautophagy.

C. Dall'Armi, A. Hurtado-Lorenzo, S. V. Voronov, G. Di Paolo; Department of Pathology, Columbia University Medical Center, New York, NY

Macroautophagy (hereafter referred to as autophagy) is an essential cellular process, whereby cytoplasmic components are imported into the lumen of lysosomes for degradation. This pathway ensures the recycling of macromolecules under conditions of nutritional scarcity, the elimination of defective organelles and the clearance of long-lived proteins as well as protein aggregates. While a number of genes have been implicated in autophagy, the molecular basis for this process is still unclear. Based on growing evidence implicating intracellular signaling lipids in autophagy, we have explored the role of phosphatidic acid (PA) and specifically, phospholipase D (PLD)-derived PA in this process. PA is a major signaling lipid that has been implicated in multiple aspects of membrane trafficking. Here we show that two members of the PLD family, mammalian PLD1 and PLD2, regulate starvation-induced autophagy. We found that PLD1 and, to a lesser extent, PLD2, partially colocalize with GFP-LC3, a well-established marker for autophagosomes, following starvation-induced autophagy. Consistent with this, both PLD1 and PLD2 were found to interact with LC3 in pulldown assays. Importantly, to better understand the physiological significance of this interaction, PLD1 and PLD2 were knocked down (KD) in independent cell lines using RNA interference. Results showed that silencing of PLD1 and PLD2 in the presence or absence of bafilomycin (which blocks the acidification of autophagolysosomes and thus, their turnover) results in a strong inhibition of autophagy, based on the reduced presence of LC3-positive structures in KD cells observed by light microscopy and on decreased levels of LC3-II (i.e., the lipidated form of LC3) based on a Western blot analysis. Altogether, our results reveal a novel signaling role for mammalian PLD in autophagy and implicate its enzymatic product, PA, in the regulation of membrane dynamics underlying this phenomenon.

Extracellular Matrix and Cell Behavior II (2328 – 2344)

2328/B35

Tenascin-W Has Anti-adhesive Properties In Vitro and Is Upregulated in Fibroblasts Isolated from the Tenascin-C Knockout Mouse.

R. P. Tucker[1], F. Brellier[2], E. Martina[2], M. Brown-Luedi[2], R. Chiquet-Ehrismann[2]; [1]University of California Davis, Davis, CA, [2]Friedrich Miescher Institute, Basel, Switzerland

Tenascins are glycoproteins found in the extracellular matrix of chordates. The best studied is tenascin-C, which promotes cell motility and is upregulated in many solid tumors. Tenascin-C has anti-adhesive properties: cells cultured on fibronectin lose their focal adhesions when it is added to the medium, and cells remain rounded when it is used as a substratum. Previously, we showed that tenascin-W is found in a subset of the tenascin-C-rich extracellular matrix of mouse embryos, and that tenascin-W is upregulated in the stroma of some solid tumors. To determine if tenascin-W shares basic anti-adhesive properties with tenascin-C, C2C12 (murine myoblast-derived) cells were cultured on tissue culture plastic coated with various substrata. C2C12 cells cultured for 90 min on fibronectin followed by adding tenascin-W to the medium for 30 min have significantly fewer ($p < 0.01$) vinculin-positive focal adhesions than C2C12 cells cultured on fibronectin for 2 hr without tenascin-W in the medium. Under these conditions, cell area measurements indicated that the C2C12 cells were also significantly more rounded ($p < 0.05$) than the fibronectin-only controls. Similarly, C2C12 cells cultured for 2 hr on tenascin-W-coated substrata, or on fibronectin mixed with tenascin-W, were significantly more rounded ($p < 0.05$) than C2C12 cells cultured on uncoated tissue culture plastic or on fibronectin alone. The tenascin-C knockout mice have subtle developmental and behavioral phenotypes. To determine if tenascin-W has the potential to be upregulated in the tenascin-C knockout mice, the expression of tenascin-W by fibroblasts isolated from tenascin-C knockout embryos was studied by immunoblotting. The mouse embryo fibroblasts isolated from tenascin-C knockout mice make more tenascin-W than fibroblasts from wild-type mouse embryos. Thus, tenascin-W shares basic anti-adhesive properties with tenascin-C, and it may be able to compensate, at least in part, for the loss of tenascin-C in the tenascin-C knockout mouse.

2329/B36

A Role for Microfibril-Associated Glycoprotein-2 in Wound Repair.

C. R. Francom[1], T. Broekelmann[2], R. Knutsen[2], R. P. Mecham[2], B. C. Trask[1]; [1]Zoology, Weber State University, Ogden, UT, [2]Cell Biology and Physiology, Washington University School of Medicine, St. Louis, MO

The principle components of 12nm microfibrils are members of the fibrillin family, although other proteins, including microfibril-associated glycoproteins-1 and -2 (MAGP-1 and -2), may contribute to the function of these structures. Both fibrillin and MAGP-1 have been shown to interact with members of the TGF-β family of growth factors and modulate their activity in vitro and in vivo. To investigate whether MAGP-2 might also interact with growth factors, the binding properties of recombinant MAGP-2 were studied using surface plasmon resonance. Solid phase binding assays were also performed to identify insoluble binding partners. These studies revealed novel interactions including an MAGP-2 self-association and an interaction with the active forms of TGF-β family members. The association between MAGP-2 and TGF-β1 is of particular interest given the biological importance of binding between the latent form of this growth factor and fibrillin. Because a delay in wound closure has been observed in MAGP-1 deficient mice, cutaneous wound-healing assays were performed in MAGP-1/MAGP-2 null (DKO) mice and wound closure rates relative to WT and MAGP-1 knockout animals were compared. Although no obvious abnormalities were evident in histological sections of skin from either of the MAGP-null animals, a significant lag in the initial rate of wound closure was observed in both groups of transgenic mice. MAGP DKO animals displayed a greater initial delay followed by quicker overall wound closure when compared to MAGP-1 null and WT animals. These studies suggest a biological role for MAGP-2 different from that of MAGP-1 in wound repair.

2330/B37

Murine γherpesvirus-68 Infection of S100A4 Overexpressing Mice Induces Pulmonary Vascular Disease Associated with Altered Microrna Expression.

Y. Kim, E. Spiekerkoetter, L. Wang, M. Rabinovitch; Pediatric Cardiology, Stanford University, Stanford, CA

Evidence of heightened expression of S100A4, a metastasis gene, and of a prior infection with human γherpesvirus is observed in neointimal pulmonary arterial (PA) lesions associated with PA hypertension. Consistent with these features, our laboratory reported that mice overexpressing S100A4, but not C57 wild-type mice, develop PA neointimal lesions following infection with murine γherpesvirus-68. These neointimal lesions progressed in severity during the reactivation phase of viral replication from 3 to 6 mo post infection, and affected about 40% of the PAs. The appearance of the PA neointimal lesions was preceded by pulmonary serine elastase activity at 1 wk and 3 mo post viral infection, and was associated with on-going elastolysis up to 6 mo post viral infection. We, therefore, hypothesized that in S100A4 mice the virus increases AML1, a transcription factor for serine elastase, due to greater viral-mediated repression of miR-106a that targets the 3'UTR of AML1. In contrast to our hypothesis, miR-106a increased after viral infection, but the elevation was significant only in the C57 mice. This was associated with impaired mRNA translation of AML1 in C57 mice ($p<0.01$) that could account in part for the lower elastase activity observed. To identify an alternative mechanism responsible for the susceptibility of S100A4 mice to viral infection-induced PA neointimal lesions, we used a platform of microRNA microarrays followed by RT-qPCR. We found that viral infection elevated the expression of miR-155 in both C57 and S100A4 mice, but that the level of miR-155 was significantly lower in S100A4 mice ($p<0.01$) at baseline, and at 1 wk and 6 mo post viral infection. TargetScan predicts 254 mRNA targeted by this miR-155. Among these, an mRNA of osteoglycin, a gene elevated in neointimal lesions in balloon injury model, was significantly elevated in S100A4 vs. C57 mice infected with the virus for 6 mo, as assessed by microarray analysis. We propose that the inability of S100A4 mice to induce miR-155 to the level found in C57 mice may result in heightened expression of osteoglycin, mediating in the pathogenesis of the the viral infection-induced pulmonary vascular disease.

2331/B38

Study of Cell Adhesion and Directed Cell Migration on Engineered Extra Cellular Matrices.

S. Sarkar[1], J. Adams[2], V. Misra[1]; [1]Electrical and Computer Engineering, North Carolina State University, Raleigh, NC, [2]BioForce Nanosciences, Inc., Ames, IA

Cells have an intricate relationship with the extra cellular matrix (ECM) proteins. The spatial organization of ECM proteins dictates the adhesion, spreading and migration of cells as these proteins provide vital cues to the receptors present on the surface of the cell. The complex nature of the cell-ECM signaling can be understood better by engineering surfaces with spatial control of ECM proteins that provides controlled environment for studying cell migration. In this work, spatially-defined microarrays of fibronectin (ECM protein) with defined domain size (10-25 μm) have been designed using the Nano eNablerTM (Bioforce Nanosciences, Inc., Ames, IA) and these domains are separated by areas that are non-adhesive to cells. The adherence and spread of the NIH 3T3 cells cultured on these engineered surfaces have been studied. The cell binding to the substrata is dependent on several factors including, time, domain size of ECM proteins and the separation of the non-adhesive region present in-between the domains. The cell adherence has been initially restricted to the patterns created by the ECM proteins however, with time the cells generate their own proteins on the substrate and diffuse away from the initial pattern. These patterned surfaces have been further used for studying directed cell migration. In vivo, a variety of cells respond to endogenous direct current electric fields (dc EF) that occur in the form of transcellular epithelial potential (TEP), which has significant relevance to directed cell migration and wound healing. The application of such small dc EFs in vitro provides electrical signals to cells in order to have a directional migration that strongly depends on the EF gradient (galvanotaxis). The cells on the NeN engineered surfaces have been subjected to electric field to accomplish directed cell movement and study the effect of ECM protein on this directional cell migration.

2332/B39

Mimicking Cellular Environments: Cells on Elastic Nanopatterned Substrates.

I. Louban[1,2], R. Fiammengo[1,2], D. Aydin[1,2], C. Selhuber-Unkel[3], J. P. Spatz[1,2]; [1]Biophysical Chemistry, University of Heidelberg, Heidelberg, Germany, [2]New Materials and Biosystems, Max Planck Institute for Metal Research, Suttgart, Germany, [3]Optical Tweezers Group, Niels Bohr Institute, Copenhagen, Denmark

The design of the extracellular matrix (ECM), e.g. compliance and biochemical functionality, governs a wide range of cellular properties and functions. Its influence on cell behaviour such as proliferation, migration and adhesion is not conclusively evaluated by biophysical means. The last years, hydrogels based on poly(ethylene glycol) diacrylate (PEG-DA) have been developed to serve as synthetic ECM analogues with adjustable mechanical properties. Their Young's moduli (E_Y) span more than four orders of magnitude: from petri-dish-like, stiff surfaces (E_Y=6 MPa) to soft, gelatinous substrates with E_Y=0.6 kPa. Since PEG-DA features protein and consequentially cell repellent properties, the hydrogel surface has to be modified to provide bioactivity. Extended or microstructured gold-nanoparticle arrays, manufactured by block copolymer micellar nanolithography, could be transferred to the hydrogel surface providing single anchor points for

biofunctionalization. The interparticle distance (30 nm≦Δ L≦200 nm) and density on the substrate can be varied independently from the

hydrogel type and rigidity. To promote integrin mediated cell adhesion of rat embryonic fibroblasts, extended gold nanopatterns on the surface of PEG-DA hydrogels were functionalized with a cRGDfK peptide specific for $\alpha_V\beta_3$ integrin. First, the effect of variation of interparticle spacing on cellular behavior was investigated on hard substrates (E_Y>100 kPa). We performed atomic force spectroscopy and live cell imaging to quantify cellular adhesion to these surfaces. We could demonstrate a strong increase in detachment force and spreading area on substrates having an interparticle spacing of 50 nm compared to those with 90 nm. Than, substrate compliance was tuned whereas their interparticle distance was kept at 50 nm. This reveal a significant decrease in spreading area on soft substrates (E_Y<10 kPa). Additionally, substrate compliance and interparticle spacing were varied at the same time. Results from the experiments are determined as a function of two independent parameters: hydrogel stiffness and ligand distance. The latter enables the quantification of cooperative processes at the molecular scale, which govern cell adhesion phenomena.

2333/B40

Roles of Collagen Crosslinking and ECM Remodeling in Mammary Tumor Malignant Transformation.

H. Yu[1], K. Levantal[2], L. Kass[1], J. Erler[6], M. Yamauchi[5], R. Wells[4], D. Gasser[3], V. Weaver[1,2]; [1]Surgery, UCSF, San Francisco, CA, [2]Institute for Medicine and Engineering, University of Pennylvania, Philadelphia, PA, [3]Genetics, University of Pennsylvania, Philadelphia, PA, [4]Medicine, University of Pennsylvania, Philadelphia, PA, [5]Dentistry, University of North Carolina at Chapel Hill, Chapel Hill, NC, [6]Section of Cell and Molecular Biology, Institute of Cancer Research, London, United Kingdom

In Vivo, cells are maintained in mechanical balanced microenvironments. Previously, we showed ECM stiffness alter cell proliferation, survival and polarity via integrin clustering, focal adhesion maturation, and cell-generated force. Increased tissue stiffness, changes of ECM (e.g. collagen) remodeling and ECM remodeling enzymes (such as MMPs, lysyl oxidase LOX) are strongly associated with breast cancer progression. We therefore hypothesis ECM remodeling affects tumor progression via increasing tissue stiffness. Since crosslinking of collagen I increase its mechanical strength, we tested if collagen crosslinking by Lysyl Oxidase (LOX) etc. affects tumor progression. We xenografted MCF10AT.DCIS into 3T3Lox+/+ pre-conditioned mammary fat-pad; we also inhibited the LOX activity in MMTV-Her2/Neu mice (with pharmacological inhibitor and function-blocking antibody). We found collagen I bundle and linearization around MMTV-Her2/Neu tumors, and increased stiffness of the tumor were partially corrected by BAPN/mAb treatment; importantly, DCIS cells became invasive in the 3T3.Lox preconditioned fat pad; tumor incidence rate and progression rate of MMTV-Her2/neu mice were reduced by BAPN and LOX-mAb treatment. We found in the LOX inhibited her2/neu tumors, b1-integrin, P130cas, FAKpY397 and PI3Kinase activity decreased while PTEN levels increased. We tested and confirmed ECM stiffness can modulate PTEN level and PI3K activity in the culture system. Thus, collagen crosslinking and substrate stiffness can modulate oncogene effects through PTEN and integrin dependent pathways and thus affect breast cancer progression.

2334/B41

The Interplay between Matrix Translocation and Cell Migration in 3D Collagen Matrices Is Modulated by Collagen Matrix Density.

M. Miron-Mendoza, J. Seemann, F. Grinnell; Cell Biology, UT Southwestern Medical Center, Dallas, TX

In nested collagen matrices, human fibroblasts migrate from dermal equivalents into outer collagen matrices, and fibroblast tractional force can couple to either cell migration or collagen flow (Mol Biol Cell, 19:2051, 2008). Here, we prepared nested collagen matrices in with different outer matrix collagen concentrations and measured cell migration and collagen flow depending on whether nested matrices were floating in culture medium or restrained on culture surfaces. Under restrained condition, cell migration predominates over collagen flow at all collagen concentration tested. Under floating conditions, with 1 mg/ml outer matrices, collagen flow predominates over cell migration; whereas with 4 mg/ml outer matrices, migration predominates over collagen flow. Using 3D reconstruction, we observed that with 1.5 mg/ml outer matrices and restrained conditions, migrating cells accumulate preferentially in the direction of the restraining culture surface. With 4 mg/ml outer matrices, no preferential accumulation occurs. Time-lapse videos demonstrate that differences in cell migration trajectories account for the differences in collagen accumulation. We speculate that at 1 mg/ml, collagen fibrils in the outer matrix cannot resist the tractional force exerted by cells unless the fibrils are restrained. However at 4 mg/ml, collagen fibril organization becomes strong enough to resist the tractional force exerted by cells. Consistent with this hypothesis, fibroblasts incubated on 1 mg/ml collagen matrices spread in dendritic morphology and exhibit low cell-matrix tension as indicated by absence of stress fibers and focal adhesions; whereas cells on 4 mg/ml exhibit lamellar spreading and develop stress fibers and focal adhesions. Rheometer measurements showed that over the range of 1 to 4 mg/ml, dynamic moduli of collagen matrices increased from ~5 to ~75 Pa. Our findings suggest that cell migration in nested matrices requires the ability of cells to develop high cell-matrix tension, and matrix stiffness may be a key parameter regulating the cellular response.

2335/B42

Cytoskeletal Dynamics during Endothelial Cell Morphogenesis on a Compliant Extracellular Matrix.

K. Lyle, J. Corleto, T. Wittmann; Cell and Tissue Biology, University of California, San Francisco, San Francisco, CA

Sprouting of new capillaries from existing vessels, or angiogenesis, is required for normal development and contributes to the development of cancer and arthritis. In order to form capillaries, endothelial cells migrate and undergo morphological changes in response to growth factors and the surrounding extracellular matrix (ECM). Basic elements of angiogenesis such as cell morphology changes, migration, and cell-cell interactions are mimicked in an in vitro angiogenesis assay using compliant ECMs such as Matrigel™ or collagen gels. Although the actin and microtubule cytoskeletons have been studied extensively on two-dimensional rigid substrates, their dynamics and functional integration are not well understood in more physiological three-dimensional environments. Using high-resolution time-lapse microscopy, we are analyzing cell morphology and cytoskeletal dynamics of human umbilical vein endothelial cells (HUVECs) in Matrigel™ substrates. We found that endothelial cells rapidly respond to Matrigel™ and form long, thin extensions that pull on and deform the ECM and eventually mature to form robust contacts with opposing cells. These long extensions contain a dense array of microtubules that persistently grow to the extension tip. We are using a combination of small molecule inhibitors and exogenously-expressed proteins to determine the contribution of the actin and microtubule cytoskeletons during cell morphogenesis on Matrigel™. Low concentrations of the microtubule-targeting drug nocodazole inhibit the formation of cell extensions and robust capillary like structures, and blebbistatin, an actinomyosin contractility inhibitor alters the dimensions of the capillary like network. These data indicate that microtubules regulate endothelial cell morphology changes in response to compliant ECMs by either stabilizing cell extensions or promoting extension elongation.

2336/B43

Deconstructing the 3rd Dimension: How Matrix Dimensionality Promotes Survival.

C. Frantz[1], J. Friedland[2], J. N. Lakins[1], W. Liu[2], J. Chernoff[3], M. A. Schwartz[4], C. S. Chen[2], D. Boettiger[2], V. M. Weaver[1, 2]; [1]Surgery and Center of Bioengineering and Tissue Regeneration, University of California, San Francisco, San Francisco, CA, [2]Bioengineering and Institute for Medicine and Engineering, University of Pennsylvania, Philadelphia, PA, [3]Fox Chase Cancer Center, Philadelphia, PA, [4]Biochemistry, University of Virginia, Charlottesville, VA

Cancer metastasis depends upon the dissemination of isolated tumor cells into a three dimensional (3D) parenchyma and their subsequent survival in distal tissues. At present information about the molecular mechanisms regulating cell survival have largely been deduced by studying the behavior of cells on two dimensional (2D) matrices. To clarify how tumor cells might survive within a 3D microenvironment we assessed the effect of cell shape and matrix spreading and integrin-dependent adhesion on the survival of isolated mammary epithelial cells (MECs) in 2D versus 3D. Provocatively, we could show that MEC viability is sustained by laminin ligation of α6β4 integrin and Rac-dependent Pak activity in round, non-spread MECs in 3D, but not in 2D. Conversely, we determined that laminin-dependent growth and survival of MECs depends upon β1 integrin ligation and ERK and PI3Kinase activity in spread MECs in 2D, but not in 3D. Such differential survival mechanisms could be attributed to enhanced GTP loading of Rac and Arf6 and reduced Rac-dependent ROS and MMP activation in 3D. Experiments revealed that elevated Arf6 GTPase activity promotes MEC survival by enhancing Rac-Pak signaling and reducing Rac-NADPH-ROS production. Because MECs interacting with laminin in 3D showed pronounced changes in cytoskeletal organization and cell size and shape, studies are in progress to test whether matrix presentation could modulate Arf6-dependent cell fate by influencing membrane curvature, protein trafficking or actin remodeling and if so how. (Supp: 7R01CA078731-07, W81XWH-05-1-330 and RS1-00449 to VMW).

2337/B44

Matrix Stiffness Directs Stem Cell Cytoskeletal Organization in 2D and 3D.

F. Rehfeldt[1,2], A. L. Zajac[1], A. E. Brown[1], S. Cai[1], D. Discher[1]; [1]Molecular/Cell Biophysics Lab, University of Pennsylvania, Philadelphia, PA, [2]Department of Physics, University of Goettingen, Goettingen, Germany

It is increasingly evident that physical cues such as matrix elasticity E are as important to cell behavior as soluble biochemical cues. Recently, human mesenchymal stem cells (hMSCs) from bone marrow have been found to respond to matrix elasticity by differentiation toward various lineages depending on the Young's modulus (E ~ 1 - 34 kPa) of collagen-coated polyacrylamide (PA) substrates. We present a method to finely tune the Young's modulus E of cross-linked hyaluronic acid based biocompatible hydrogels over a wide, physiologically relevant range and show the applicability for hMSC culture in two and three dimensions, demonstrating that cellular mechano-sensitivity is a general feature and is not material dependent. The dependence of cell shape and cytoskeletal arrangement on the matrix elasticity is quantified in 2D and 3D and the impact on the morphology of the nucleus is described. These well-defined biocompatible hydrogels will allow for quantitative dissection of cues from dimensionality and matrix elasticity and open up new opportunities for potential in vivo applications as well as more sophisticated biomimetic extracellular matrix models.

2338/B45

Pleiotropic Effects of Nuclear Co-Repressor 2 (N-CoR2) on Breast Cancer Progression.

K. K. Tsai[1,2], P. Y. Chu[1], J. N. Lakins[3], V. Weaver[3]; [1]National Institute of Cancer Research, National Health Research Institute, Tainan, Taiwan, [2]Graduate Institute of Clinical Research, Taipei Medical University, Taipei, Taiwan, [3]Department of Surgery and Center for Bioengineering and Tissue Regeneration, University of California, San Francisco, San Francisco, CA

Epigenetic changes, including DNA methylation, histone modifications and chromatin remodeling, play a pivotal role in the initiation as well as evolution of malignant tumors. We previously showed that an important epigenetic regulator, N-CoR2 (Nuclear corepressor-2), mediates multidrug resistance and contributes to poor therapeutic outcome of human breast cancers. We now demonstrate a previously unidentified function of N-CoR2 in tumor progression, which is distinct from its role in cell death resistance. We could show that N-CoR2 enhances the proliferation of neoplastic mammary epithelial cells (MECs) and inhibits their invasion through a reconstituted basement membrane in vitro. Using gene expression profiling, we found that N-CoR2 represses the expression of several genes implicated in breast cancer invasion and metastasis, including fibronectin 1 (FN1), thrombospondin-1 (THBS1) and matrix metalloproteinase-1 (MMP1). Forced expression of these proteins in N-CoR2-expressing neoplastic MECs partially restored their invasive capacity in vitro. Analogous to its ability to induce cell death resistance, N-CoR2 transcriptionally represses pro-invasive gene expression by modulating the activity of histone deacetylase 3 (HDAC3) as illustrated by the observation that the HDAC3 K449A mutant N-CoR2 failed to prevent breast cancer cell invasion or repress the expression of pro invasion genes. The finding that N-CoR2 modulates tumor cell growth, survival, treatment resistance and invasion

implies that the N-CoR2-dependent epigenetic pathway is a pleiotropic regulator of cancer progression (Supported by NSC 96-2321-B-038-004 to KKC and DOD BCRP W81XWH-05-1-330 and NIH CA078731 to VMW).

2339/B46

Over-Expression of Decorin Promotes the Differentiation of C2C12 Myoblasts.

K. Suzuki, Y. Kishioka, J. Wakamatsu, A. Hattori, T. Nishimura; Meat Science Laboratory, Graduate School of Agriculture, Hokkaido University, Sapporo, Japan

Decorin, a small leucine-rich proteoglycan, plays an important role in cellular activities through modification of growth factors such as TGF-b. Decorin also acts as a signaling molecule to cells through EGFR or IGF-IR. We reported that decorin enhances the proliferation of myoblasts through suppressing the activity of myostatin, a negative regulator of muscle mass. However, it is still unclear if decorin affects on the differentiation of myoblasts. The aims of this study were to elucidate the role of decorin in the differentiation of myoblasts and the potentiality of decorin as a signaling molecule to myogenic cells. To investigate effects of decorin on the differentiation of myogenic cells, we generated stable clonal C2C12 myoblasts over-expressing decorin. The fusion index of decorin over-expressing cells was significantly higher ($P<0.01$) at 72 h after the induction of differentiation. At 144 h, decorin over-expressing cells showed multi-giant hypertrophic myotubes. These results indicate that decorin enhances the differentiation of myoblasts. It is well known that myostatin suppresses the differentiation of myoblasts. So, we examined if decorin over-expression modulates the inhibitory action of myostatin on the differentiation. Exogenously added myostatin inhibited the differentiation of myoblasts in a dose-dependent manner in both decorin over-expressing and control cells. When 1.5 µg/ml of myostatin was added to the media, the fusion index of control cells was inhibited by 36%, whereas that of decorin over-expressing cells was inhibited by 15%. This result suggests that decorin attenuates the myostatin-induced inhibitory action to myoblast differentiation. Next, to investigate if decorin affects EGF and IGF-I signalling pathways, the phosphorylation levels of ERK1/2 and Akt in decorin over-expressing cells were examined by Western blot. The phosphorylation level of ERK1/2 in decorin over-expressing cells was equivalent to that in control cells. Akt, downstream of IGF-I, was more phosphorylated in decorin over-expressing cells as compared to control cells throughout the differentiation. This result suggests the possibility that decorin affects myoblasts differentiation through the IGF-I signaling pathway.

2340/B47

Mechanotaxis Effects on Cortical Neuron Outgrowth.

L. Matta-Norman, H. Aranda-Espinoza; Fischell Dept. of Bioengineering, University of Maryland, College Park, MD

A thorough understanding of how the mechanical properties of substrates affect neuronal behavior and response is critical in not only embryonic development, but also in the production of efficient synthetic biomaterials for neural regeneration. In particular, substrate stiffness has become a critical constituent of in vitro evaluation of neuronal viability, branching and functionality. Here, we describe the effects of substrate stiffness on neuronal processes for both single cell and cortical explants isolated from E17 rat pups. We evaluated outgrowth lengths of neuronal processes on laminin-coated polyacrylamide gels ranging in stiffness between 0.15 - 1.65 kPa, and used force traction microscopy to quantify traction forces during neuronal outgrowth within the first 72 hours of culture. First, for all stiffnesses, we identify neuronal processes as statistically longer for groups of neurons versus single cells. The difference in process extension rates between groups versus single cells was most prominent on the stiffest gel, where single cells extended with an average rate of 0.7µm/hr, while groups of cells extended processes more quickly, with an average extension of 1.28µm/hr. Second, after 48 hours of culture, we observe statistically longer processes on 1.65 kPa gels compared to all other stiffnesses. However, no statistical differences were observed when comparing outgrowth on gels less than 1 kPa in stiffness, suggesting that certain stiffnesses may be too soft for cortical outgrowth. Here, we also report an increase in traction forces as the substrate stiffness increases. We postulate that the ability to exert larger traction forces allows for increased rates in process outgrowth. Collectively, this data presents newly identified mechanotaxis effects on cortical neuron outgrowth and suggests the ability to modify process extension based on substrate stiffness.

2341/B48

Extracellular Matrix Elasticity Regulates Glioblastoma Cell Structure, Motility, and Physiology.

T. A. Ulrich[1], A. Jain[2], S. Kumar[1,2]; [1]UCSF/UC Berkeley Joint Graduate Group in Bioengineering, University of California, Berkeley, Berkeley, CA, [2]Department of Bioengineering, University of California, Berkeley, Berkeley, CA

Glioblastoma multiforme (GBM) is a high-grade malignant astrocytoma of the central nervous system associated with a mean survival time of less than one year, even with aggressive surgical care, chemotherapy, and radiotherapy. This rapid progression is due in part to the diffuse infiltration of single tumor cells into the normal brain parenchyma, which is thought to involve aberrant interactions between tumor cells and the extracellular matrix (ECM). Here, we address this problem from a mechanobiological perspective, exploring if and how the mechanical interplay between the ECM and the cellular cytoskeleton contributes to GBM tumor cell mechanics and physiology. By culturing GBM tumor cells on two-dimensional (2D) ECM substrates of defined chemistry and stiffness, we find that projected tumor cell area, cytoskeletal structure, proliferation, and motility are all strongly influenced by mechanical cues from the ECM. Parallel studies in three-dimensional (3D) spheroidal culture reveal that GBM cell invasion into the surrounding matrix depends on ECM elasticity independent of changes in protein density, and also offer insight into differential mechanisms of GBM cell migration in 2D vs. 3D. Importantly, the relationship between ECM stiffness and cell behavior in both 2D and 3D depends on Rho GTPase-mediated cytoskeletal contractility. Collectively, our results provide support for the importance of mechanobiological stimuli in regulating GBM progression.

2342/B49

The Role of Bag-1 in Endoplasmic Reticulum Stress Using a Model of Ex Vivo-Generated Cartilage.

A. E. Nugent[1,2], D. L. McBurney[1], M. M. Moran[1,2], W. E. Horton[1,2]; [1]Anatomy & Neurobiology, Northeastern Ohio Universities Colleges of Medicine and Pharmacy, Rootstown, OH, [2]Kent State University, Kent, OH

Bcl-2 associated athanogene-1 (Bag-1) is a multifunctional protein that has recently been implicated as a downstream regulator of the endoplasmic reticulum (ER) stress response in chondrocytes. The expression of Bag-1 has been demonstrated to decline *In Vivo* with age, and to be down-regulated under ER stress conditions in monolayer. Recently, our lab has characterized a model of *ex vivo*-generated cartilage using primary bovine articular chondrocytes suspended in a collagen I scaffold. The objective of this study is to determine the role of Bag-1 under homeostatic and ER stress conditions employing this model system, hypothesizing that the presence of a complex extracellular matrix (ECM) will confer protection from ER stress-inducing agents. Chondrocytes were isolated from 1-3 week old calf knees, suspended in 2.7mg/mL collagen I, and plated as 300µL 'spot cultures' (SCs) with $5x10^6$ cells per spot. SCs were grown for a period of time (1 day through 2 weeks) before being subjected to ER stressors. Additionally, SCs grown for 1 week or 2 weeks were subjected to increasing concentrations of ER stressors. Immunostaining for the ER chaperone Grp78 indicates matrix protection of SCs subjected to glucose withdrawal, but not tunicamycin (TN) or thapsigargin (TG) treatment. SCs subjected to ER stressors up-regulate Grp78 at the protein and mRNA levels even at low concentrations of TN and TG, but do not up-regulate Gadd153; suggesting that the cells are experiencing ER stress but will not terminate in apoptosis. In contrast, bovine chondrocytes in monolayer up-regulate both Grp78 and Gadd153. These results suggest that the presence of an ECM confers some protection from ER stressors, with further studies underway to determine the role of Bag-1 in this response. [This work was supported by grants from the National Institutes of Health and the Arthritis Foundation]

2343/B50

Type IV Collagen Induces Changes in the Basal Ectopic Expression in Plasmatic Membrane and Perinuclear Redistribution of the Tetraspanin CD9 in Breast Cancer Cell Lines.

L. A. Castro-Sanchez, A. Soto-Guzman, N. Navarro-Tito, T. Robledo, E. Perez Salazar; Cell Biology, CINVESTAV-IPN, Mexico, Mexico

CD9 is a member of the tetraspanin family and it is widely expressed at the membrane of several cell types, including malignant cells. CD9 can interact with a number of transmembrane proteins to promote the formation of functional complexes, and mediate biological processes as differentiation, proliferation, adhesion, migration and tumor cell invasion. Several lines of evidence suggest that CD9 is an important regulator of cell motility, because it can form non-covalent complexes with the integrin receptor family. Basement membrane is a specialized extracellular matrix and its main structural protein is type IV collagen (Col-IV) and it represents the principal obstacle in tumor cell-invasion. Several clinical studies have also shown an important prognostic value for CD9, a low CD9 expression is associated with metastasis in various cancers, including breast cancer. However, the role of Col-IV in the CD9 expression and redistribution remains to be studied. We evaluated the CD9 behavior in breast cancer cells stimulated by Col-IV. By using Western blot, flow cytometry and confocal microscopy, we demonstrated that Col-IV promotes an increase of CD9 at the cytoplasmatic membrane, reaching a maximum at 5 h, followed of internalization at large periods of time stimulation in breast cancer cells (MDA-MB-231 and MCF7). In contrast, Col-IV did not induce any increase of CD9 expression at the cytoplasmatic membrane in human breast epithelial cells (MCF10A). Furthermore, by using the inhibitor chloroquine, we found that the internalization of CD9 is a clathrin-mediated mechanism. Immunofluorescence staining of CD9 showed that Col-IV also promotes redistribution in the perinuclear region in function of the time of stimulation. In conclusion, our findings show for the first time that Col-IV induces changes in CD9 ectopic expression and redistribution in breast cancer cells, suggesting a possible role in traffic proteins and cellular migration.

2344/B51

Fibronectin Matrix Induces Embryonic Stem Cell Differentiation.

A. J. Engler[1,2], J. Schwarzbauer[2]; [1]Bioengineering, University of California, San Diego, La Jolla, CA, [2]Molecular Biology, Princeton University, Princeton, NJ

Fibronectin (FN), a major extracellular matrix (ECM) component that assembles into a 3-dimensional (3D) network, plays a significant role in the development and maintenance of most tissues. In the embryonic stem (ES) cell niche, ECM composition and 3D architecture likely contribute to the decision between self-renewal and differentiation. Over 8 days in culture, ES cells differentiating as multicellular embryoid bodies (EBs) exhibit a 10-fold drop in expression of Nanog, a self-renewal marker, as measured by a Nanog promoter-GFP transcriptional reporter. Concurrent with less self-renewal is a 3-fold upregulation in FN production as well as the onset of differentiation markers Fgf 5 (ectoderm), brachyury (mesoderm), and GATA4 (endoderm). However, FN and GATA4 appear to be temporally and spatially correlated within the EB while FN and Nanog are inversely correlated with each other. To probe any specific FN-GATA4 interaction, FN-coated surfaces and 3D fibrillar FN matrices were used as substrates for ES cells grown in monolayer culture for 8 days. ES cells on FN-coated surfaces displayed a well spread morphology but did not significantly increase their FN production. In contrast, ES cells grown on fibrillar matrices were less spread, displayed a 4-fold upregulation of FN production similar to that of EBs, and expressed GATA4 via immunofluorescent detection. Evidence also suggests a role for matrix stiffness in the self-renewal to differentiation switch as cell migration, proliferation, and even FN, Nanog, and GATA4 expression all appear matrix stiffness dependent. Though the specific molecular mechanisms still need to be elucidated, these findings suggest important temporal, spatial, and mechanical roles for FN matrix in regulation of ES cell development.

Integrins (2345 – 2370)

2345/B52

Integrin Is Required for Normal Drosophila Heart Function.

L. Ma[1], A. Bradu[2], A. Podoleanu[2], J. Bloor[1]; [1]Biosciences, University of Kent at Canterbury, Canterbury, United Kingdom, [2]Physics, University of Kent at Canterbury, Canterbury, United Kingdom

Integrins are associated with a number of cardiac diseases in both human patients and vertebrate models, including cardiac hypertrophy, ischemic cardiomyopathy and dilated cardiomyopathy. Here we exploit the *Drosophila* larval heart as a system in which to explore integrin cardiac function. The larval heart is composed of two parts, an anterior tubular aorta and a posterior cardiac region that is broad, contractile,

and which contains valves that allow directional flow of hemolymph through the organ. To explore the role of integrins and integrin-related genes in cardiac function we combine heart-specific RNAi expression with a novel label-less imaging technology called Optical Coherence Tomography (OCT) that provides direct imaging of the heart in wild type and knockdown larvae. To date we have examined the role of the PS2 integrin in larval cardiac function. RNAi constructs targeted to myospheroid or inflated (corresponding to betaPS and alphaPS2 integrin subunits) cause a similar dilated heart defect associated with a reduction in cardiac output. Moreover we have developed our OCT system to produce a "stethoscope" that can monitor both heart rate and the speed at which the heart wall contracts. Both are reduced in integrin knockdown hearts. Conclusion: integrin is required for normal *Drosophila* heart function. We are currently extending this work by 1) examining the cell biology underlying the dilated heart defect and 2) determining which downstream signalling pathways are involved.

2347/B54

Priming of α2β1 Integrin on Platelets.

R. Marjoram[1], B. Voss[2], Y. Pan[1], K. Dickeson[1], M. Zutter[1], H. Hamm[2], S. Santoro[1]; [1]Pathology, Vanderbilt University, Nashville, TN, [2]Pharmacology, Vanderbilt University, Nashville, TN

The blood platelet plays a central role in primary hemostasis at sites of vascular injury and in pathologic thrombosis. Thrombin and fibrillar collagens are two potent activators of platelets at sites of vascular injury. Human platelets express two thrombin receptors, protease-activated receptors 1 and 4 (PAR1 and PAR4) and two collagen receptors, α2β1 integrin (α2β1) and glycoprotein VI (GPVI). These receptors and their signaling mechanisms have been intensely studied, but it is still not fully known how these receptors cooperate in platelet functions. This study was designed to assess how the thrombin and collagen receptors cooperate in platelet hemostatic functions. In order to analyze α2β1-mediated adhesion of platelets, we designed an α2β1-specific collagen-related peptide (α2-CRP) that contains the amino acid sequence, GFOGER (O = 4-hydroxyproline), which α2β1 specifically binds. PAR-activating peptides specific for PAR1 (PAR1-AP) or PAR4 (PAR4-AP) were utilized to determine the contributions the two thrombin receptors make towards platelet adhesion to α2-CRP and collagen I. We demonstrate that platelet adhesion to α2-CRP is enhanced by suboptimal activation (concentrations that do not stimulate platelet aggregation) of PAR1 and PAR4 by either the PAR-APs or thrombin. Suboptimal concentrations of PAR4-AP produced a greater enhancement of platelet adhesion than PAR1-AP. Suboptimal activation of other platelet Gq-linked G protein-coupled receptors (GPCRs) produce enhanced platelet adhesion to α2-CRP. We show that the enhanced α2β1-mediated platelet adhesion is controlled by phospholipase C (PLC), since inhibition of PLC by U73122 prevents the agonist-stimulated increase in platelet adhesion to α2-CRP. This enhanced platelet adhesion is not dependent on granule secretion, activation of αIIbβ3 integrin, or on phosphoinositol-3 kinase (PI3K) activity. Use of wild-type and knock-out α2β1 or GPVI mouse platelets confirmed suboptimal PAR4 activation enhances platelet adhesion to α2-CRP and was PLC-dependent. We conclude that suboptimal activation of platelet Gq-linked GPCRs can stimulate enhanced platelet adhesion to collagens through the α2β1 integrin by a PLC-dependent priming mechanism.

2348/B55

Integrin α5β1 Enhanced Tumor Cell Invasiveness by Contractile Force Generation, but Decreased Tumor Growth.

C. T. Mierke[1], B. Frey[2], M. Fellner[1], M. Herrmann[2]; [1]Center for Medical Physics and Technology, Biophysics Group, University of Erlangen-Nuremberg, Erlangen, Germany, [2]Internal Medicine III, University Hospital Erlangen, Clinical Immunology and Rheumatology, Erlangen, Germany

The process of metastasis formation that includes cell invasion has been shown to cause malignant progression of tumors. The impact of cell mechanical properties on the malignancy of tumor cells has not been investigated systematically. Highly-invasive tumor cells expressed significantly higher amounts of the α5β1 integrin compared to weakly-invasive tumor cells. We hypothesized that the ability to generate contractile forces is a prerequisite for cell invasion and that α5β1-expression increased cell invasion through enhanced generation of these contractile forces. We analyzed whether α5β1-high or α5β1-low expressing breast carcinoma cells differ in their ability to invade into a 3-D collagen fiber matrix. Our results show that higher α5β1 expression increases cell invasiveness. We found that the increased 3-D motility of α5β1-high expressing cells can be explained by the integrin α5β1 activating traction generation. Furthermore, we analyzed whether α5β1-high and α5β1-low cells formed tumors in mice. The tumor formation and growth is impaired in α5β1-high compared to α5β1-low cells. The integrin α5β1 acts as an enhancer of cell invasiveness where contractile forces are necessary to overcome the viscous drag, but as a suppressor of primary tumor formation and growth where increased motility is rather a hindrance for cell clustering to form tumors.

2349/B56

Breast Cancer Cell Invasion Depends on A3β1 Integrin Expression.

M. Fellner, C. T. Mierke; Center for Medical Physics and Technology, Biophysics Group, University of Erlangen-Nuremberg, Erlangen, Germany

Integrins have been shown to be involved in tumor cell invasion leading to malignant progression of tumors. We analyzed the expression of the integrin receptor α3β1 on the cell surface of 24 invasive tumor cell lines and 27 non-invasive tumor cells. Invasive tumor cells expressed significantly more α3β1 integrin compared to non-invasive tumor cells. The impact of the α3β1 integrin on the malignancy of tumor cells is still contrary discussed in the literature. We hypothesized that the expression of the α3β1 integrin enhances tumor cell invasion. Using RNAi technology, we knocked-down the α3β1 integrin expression in human breast cancer cells and determined their motility in a 3-D collagen fiber network. The invasiveness expressed as invasion score (average invasion depths multiplied with the number of invaded cells per sqmm) is 5-fold decreased in α3β1 knock-down cells compared to cells treated with control siRNA. The integrin α3β1 enhances cell invasiveness and may promote the process of metastasis formation.

2350/B57

β1 Integrin Regulates Recruitment of αvβ3 Integrin to Focal Contacts in Endothelial Cells.

A. M. Gonzalez, R. Bhattacharya, J. C. Jones; Cell and Molecular Biology, Northwestern University, The Feinberg School of Medicine, Chicago, IL

Endothelial cells (ECs) that line blood vessels express a number of integrin receptors, including the αvβ3 and α5β1 heterodimers. Our research is focused on gaining a better understanding of how integrins function together to regulate cellular processes including adhesion, migration and proliferation. In this study, we demonstrate that under serum-free conditions, αvβ3 integrin fails to localize to focal contacts in ECs plated onto a fibronectin coated substrate. In contrast, when ECs, transfected with a β1 integrin siRNA, are plated onto fibronectin, αvβ3 integrin localizes robustly to focal contacts. These results suggests that ligand occupancy of β1 integrin elicits signals that inhibit αvβ3 integrin recruitment to focal contacts. Consistent with this model, β1 integrin antibody-induced clustering inhibits αvβ3 integrin-mediated adhesion of ECs to several ligands. Moreover, we demonstrate that antibody-induced clustering of β1 integrin enhances protein kinase A (PKA) activity and increases β3 integrin serine phosphorylation. Addition of H-89, an inhibitor of PKA, abrogrates the effect of β1 integrin antibodies on β3 integrin serine phosphorylation and cell adhesion. We have also examined the involvement of PKA in αvβ3 integrin recruitment to focal contacts. Recruitment of αvβ3 integrin to focal contacts in ECs transfected with β1 integrin siRNA and plated on fibronectin is inhibited by treatment of the cells with forskolin, an activator of PKA. Together these results suggest that in ECs, ligand occupancy by β1 integrin activates signals that maintain αvβ3 integrin in a low affinity, inactivate state. These findings may explain why there is little "active" αvβ3 integrin in mature blood vessels, since in the ECs lining such blood vessels, β1 integrin is presumably ligated while PKA is active leading to a decrease in αvβ3 integrin-ligand binding.

2351/B58

Talin Regulates Integrin αiibβ3 Avidity, Not Affinity, in CHO Cells.

T. A. Bunch; Molecular and Cellular Biology, University of Arizona, Tucson, AZ

Talin is a cytoplasmic protein that has the capacity to bind to the cytoplasmic domains of integrin beta subunits. Numerous reports indicate that talin binding to the αIIbβ3 integrins activates them. In these reports activation is commonly defined as changing the conformation of the extracellular domains of the individual integrin subunits so that the integrins have a higher affinity for their extracellular ligands. A recent examination of Drosophila PS2 integrin affinity regulation by talin found no similar effect and it was concluded that integrin affinity regulation by talin is different in invertebrates [Helsten et al., 2008. Mol Biol Cell 19(8):3589-98]. Closer examination of the procedures used to measure integrin αIIbβ3 affinity in the most common experimental paradigms suggested that these experiments measured integrin avidity and not affinity. I have reproduced the experiments demonstrating that talin head F2-F3 (FERM domains 2 and 3) overexpression increases integrin binding to a multivalent ligand, the IgM ligand mimetic PacI. No increase in binding is observed when the same ligand is presented in the monovalent form (and not clustered by the addition of secondary antibodies during the binding phase of the assay). Assays currently reported in the integrin literature often do use monovalent PacI, but secondary antibody is added together with the monovalent PacI. This is expected to render the ligand multivalent. Thus, the affinity of human integrin αIIbβ3 expressed in CHO cells, like the Drosophila PS2 integrin, is not affected by talin head overexpression. Mn2+ does strongly increase the affinity of αIIbβ3 for monovalent PacI. The D723>R activating mutation of the β3 cytoplasmic domain, exerts its effect predominantly through an increase in avidity, though there may be a small effect on affinity as well. These results mark a significant shift in the model for talin regulation of integrin activation. Using this new experimental paradigm, I also present results, from experiments currently in progress, that re-examine integrin affinity changes reported to be induced by the activation of human platelets.

2352/B59

RSK2 Modulates Integrin Activation.

J. E. Gawecka[1], M. Matter[2], J. W. Ramos[1]; [1]Department of Natural Products and Cancer Biology, Cancer Research Center of Hawaii, Honolulu, HI, [2]Department of Cell and Molecular Biology, John A. Burns School of Medicine, University of Hawaii at Manoa, Honolulu, HI

Dynamic regulation of integrin affinity for ligand (activation) is important in a broad array of cellular functions including adhesion, migration, signal transduction, and the assembly of extracellular matrices. Our major objective is to understand the molecular mechanism by which the ERK MAP kinase pathway modulates integrin activation. RSK2 is a substrate of ERK and can mediate many of the biological functions of the Ras/ERK MAP kinase pathway including cell survival, proliferation and transcription. RSKs are large kinases (90kDa) consisting of two kinase domains. The regulation of RSK activity is complex and requires phosphorylation at several sites including auto-phosphorylation. RSK2 has many substrates including cytoskelatal proteins such as the actin-binding protein, Filamin A. Both Filamin A and Talin bind to integrin beta tails in addition to actin. Indeed Talin binding activates integrins. Here we show that activated RSK2 inhibits integrin activation and fibronectin matrix assembly in fibroblasts. Moreover a dominant-negative construct of RSK2 blocks H-Ras signaling to integrins. RSK2 alteration of integrin activation is reflected in changes in integrin β-tail binding to Filamin A and Talin. These results indicate a heretofore unknown function of RSK2 in modulating integrin activation and provide insight into a novel mechanism by which the ERK MAP kinase pathway can regulate integrins.

2353/B60

Distinct Engagement of Integrin αmβ2 by Its Ligands Controls Neutrophil Apoptosis.

E. Pluskota, D. Szpak; Molecular Cardiology, Cleveland Clinic, Cleveland, OH

Neutrophils (PMNs) undergo spontaneous apoptosis, but their survival can be extended during inflammatory responses. It has been reported that integrin $\alpha_M\beta_2$ can either delay or accelerate PMN apoptosis. Objective of this study was to delineate the mechanisms by which this integrin can support such diametrically opposed responses. The effects of closely related $\alpha_M\beta_2$ ligands, Plg and angiostatin Ang(1-4), derived from Plg, as well as Fg and its two derivative $\alpha_M\beta_2$ recognition peptides, P1 and P2C on PMN apoptosis were elucidated utilizing analysis of caspases activation and Annexin V binding. Plg, Fg, and P2C suppressed PMN apoptosis (20-30% ± 4-10% AnnexinV$^+$ cells), while Ang(1-4) and P1 failed to do so. Moreover, engagement of $\alpha_M\beta_2$ with Plg, Fg and P2C, but not with Ang(1-4) and P1, resulted in $\alpha_M\beta_2$ clustering and activation of Akt and ERK1/2, which were critical in PMN survival. Based on adhesion and soluble ligand binding assays utilizing K562

cells transfected with $\alpha_M\beta_2$ or its individual α_M or β_2 subunits, and purified recombinant receptor and its constituent chains, we found that the prosurvival ligands (Plg, Fg and P2C) were recognized by both integrin subunits, while Ang(1-4) and P1 interacted preferably with the α_M subunit. Importantly, the prosurvival ligands activated Akt and prolonged life of K562 cells expressing both integrin subunits and did not delay apoptosis of cells bearing its individual subunits. In addition, both α_M and β_2 cytoplasmic tails are crucial in transmission of prosurvival signal, since the protective effect of prosurvival ligands is lost in K562 cells expressing $\alpha_M\beta_2$ with cytoplasmic tail truncations in either subunit. In conclusion, we demonstrate that different $\alpha_M\beta_2$ ligands can exert different effects on PMN apoptosis and provide a mechanism for these differential effects: engagement of both $\alpha_M\beta_2$ integrin subunits with cytoprotective ligands is prerequisite to the receptor clustering and subsequent transmission of pro-survival signals, while ligands which engage primarily the α_M subunit can not induce the pro-survival responses and do not delay PMN apoptosis.Thus, subtle balance of the $\alpha_M\beta_2$ ligands may be an important checkpoint in resolution of inflammation.

2354/B61

Activators of Vinculin Increase Integrin-Mediated Cell Adhesion.

E. S. Nelson[1,2], A. W. Folkmann[1], K. DeMali[1,2]; [1]Biochemistry, University of Iowa, Iowa City, IA, [2]Interdisciplinary Graduate Program in Molecular and Cellular Biology, University of Iowa, Iowa City, IA

Sites where cells adhere to the extracellular matrix are rich in integrins, the major cell surface adhesion receptors. Integrins are coupled to the actin cytoskeleton by a series of actin-binding proteins including vinculin. Maintenance of a connection to the underlying actin cytoskeleton is critical for integrin function and interruptions in this linkage contribute to the formation and progression of numerous diseased states including cancer, leukocyte adhesion deficiency, rheumatoid arthritis, heart disease, and some muscular dystrophies. In spite of the critical role of integrins, effective targets for manipulating integrin function are still lacking. We have recently identified a short vinculin activating peptide that increases integrin-mediated adhesion to fibronectin or collagen. The increased adhesion is both vinculin- and integrin-dependent. An analysis of the vinculin activating peptide reveals that it is comprised of three vinculin binding sites. Using a series truncation mutants we have mapped the residues sufficient for binding vinculin. We find that any one of the three binding sites in the vinculin activating peptide is sufficient for vinculin binding, co-localization with vinculin in focal adhesions, and increased integrin-mediated adhesion. We are investigating the mechanism by which the vinculin activating peptide increases adhesion with an emphasis on how it regulates integrin activity. These studies provide novel insight into how vinculin activation regulates integrin function and will open up novel therapeutic possibilities.

2355/B62

Independent Contributions of Affinity, Lateral Mobility and Clustering to β2 Integrin-Mediated Adhesion under Flow Conditions.

T. Yu[1], X. Wu[1], K. Gupta[1], D. Kucik[1,2]; [1]Pathology, University of AL at Birmingham, Birmingham, AL, [2]Pathology and Clinical Laboratory, VA Medical Center, Birmingham, AL

β2 integrins on leukocytes increase their adhesiveness both by affinity changes and avidity modulation. Avidity modulation, defined as an increase in adhesiveness independent of integrin conformation changes, is usually thought to occur by rearrangement of the integrins into clusters. Early on, however, it was predicted that not only rearrangement, but also lateral mobility of the integrin might increase avidity by increasing receptor-ligand encounters, thereby increasing the kinetics of ligand binding. Increased integrin lateral mobility (diffusion) upon activation of leukocytes has been demonstrated by multiple labs for several leukocyte types, but whether it is important in itself, or just constitutes a mechanism for integrin clustering, remains unclear. Integrin lateral mobility can be induced by a variety of agents, both physiological and pharmacological, but cytochalasin D, used in very low concentrations (0.1-0.3 μg/ml), induces integrin mobility with minimal effects on other cell functions and is often used to activate β2 integrin avidity. Diffusion measurements have confirmed that integrin mobility continues on cytochalasin D-treated cells, even after clustering. In order to determine whether continued integrin motion after clustering might play a role in adhesion activation, we fixed cells after cytochalasin D-induced integrin clustering, thereby freezing integrin motion. We then compared adhesive properties to unfixed, cytochalasin D-treated cells using a flow-chamber adhesion assay. At a physiological shear stress, adhesion was equivalent for fixed and unfixed cells except following CD, which increased adhesion for both. However, fixed-cell adhesion was less than that for unfixed cells, even though the degree of integrin clustering was indistinguishable by confocal microscopy. For both live and fixed cells, cytochalasin D increased adhesion without inducing a high affinity conformation, because integrin affinity could still be increased by treatment with Mn++. A simple explanation for our results is that fixation stops integrin motion on fixed cells, but integrins continue to diffuse on unfixed cells, increasing the kinetics of integrin/ICAM-1 interactions and enhancing adhesion.

2356/B63

Plasminogen Activator Inhibitor-1 Regulates Integrin αvβ3 Expression and Autocrine TGFβ Signaling.

B. S. Pedroja, L. E. Kang, A. O. Imas, A. M. Bernstein; Ophthalmology, Mount Sinai School of Medicine, New York, NY

Fibrosis is characterized by 1) elevated and persistent TGFβ signaling resulting in the accumulation of extracellular matrix (ECM) and 2) an increase in plasminogen activator inhibitor (PAI-1) expression. PAI-1 induces the internalization of uPA/uPAR (urokinase plasmingen activator/ receptor) and integrin αvβ3 from the cell surface. Since increased αvβ3 expression correlates with increased and sustained TGFβ signaling, we hypothesized that aberrant PAI-1-mediated αvβ3 endocytosis could initiate an autocrine-loop of TGFβ activity. To investigate this mechanism, PAI-1 WT and KO mouse embryonic fibroblasts were compared. In PAI-1 KO cells endocytosis of αvβ3 was dramatically reduced, leaving αvβ3 in greatly enlarged focal adhesions, similar to the enlarged focal adhesions that were seen in WT cells transfected with PAI-1 siRNA. TGFβ signaling was significantly enhanced in PAI-1 KO cells as demonstrated by the increased number of nuclei with SMAD 2/3 in KO cells and a 2.9-fold increase in TGFβ activity that correlated with dramatically increased total and cell-surface expression of αvβ3 and TGFβRII. As expected, PAI-1 KO cells demonstrated unregulated PA activity (and therefore unregulated plasmin). However, plasmin was only partially responsible for the increased TGFβ activity, since plasmin inhibition decreased TGFβ activity by only 25%. Consistent with the proposed connection between αvβ3 expression and TGFβ activity, an αvβ3-specific cyclic peptide (GpenGRGD) inhibited TGFβ

activity by 59%, compared to a 23% decrease using a control GRGDNP peptide. Finally, detectable collagen, a marker for fibrosis, was increased 1.6 fold in KO over WT cells. These data suggest that PAI-1-mediated regulation of αvβ3 integrin is critical for the control of TGFβ signaling and the prevention of fibrotic disease.

2357/B64

Del-1 (Developmental Endothelial Locus-1) Is an Endogenous Inhibitor of Leukocyte-Endothelial Adhesion Limiting Inflammatory Cell Recruitment.

E. Choi[1], E. Chavakis[2], M. Czabanka[3], H. Langer[1], L. Fraemohs[4], M. Economopoulou[5], R. Kundu[6], C. Gahmberg[7], M. Udey[8], P. Vajkoczy[3], T. Quertermous[6], S. Dimmeler[2], C. Weber[4], T. Chavakis[1]; [1]Experimental Immunology Branch, NIH/NCI, Bethesda, MD, [2]Molecular Cardiology, J.W. Goethe University Frankfurt, Frankfurt, Germany, [3]Department of Neurosurgery, Charite Universitätsmedizin Berlin, Berlin, Germany, [4]Institute for Molecular Cardiovascular Research, RWTH University Hospital, Aachen, Germany, [5]Laboratory of Cellular Oncology, NIH/NCI, Bethesda, MD, [6]6Division of Cardiovascular Medicine, Stanford University School of Medicine, Palo Alto, CA, [7]Division of Biochemistry, Faculty of Biosciences, University of Helsinki, Helsinki, Finland, [8]8Dermatology Branch, NIH/NCI, Bethesda, MD

Leukocyte recruitment to the sites of infection or inflammation requires a multistep process of adhesive events. While numerous players promoting leukocyte-endothelial interactions have been well characterized, functionally relevant endogenous negative regulators / inhibitors of the leukocyte adhesion cascade have not been identified. Here, we identified the endothelial-derived secreted molecule, developmental endothelial locus-1 (Del-1), as an endogenous anti-adhesive factor that interferes with LFA-1-dependent leukocyte-endothelial adhesion. A specific interaction of Del-1 with leukocyte integrin LFA-1 was detected, as evidenced by (i) direct binding of recombinant Del-1 to the recombinant ligand-binding I-domain of LFA-1 locked in the open conformation, (ii) by studying adhesion of LFA-1-proficient and LFA-1-deficient leukocytes to immobilized Del-1, (iii) as well as by performing adhesion of alphaL-transfected and vector-transfected Jbeta2.7 cells to Del-1. Despite being a ligand for LFA-1, Del-1 did not promote neutrophil adhesion under physiologic flow conditions. In addition, Del-1 competed with ICAM-1 for binding to LFA-1 on mouse leukocytes and interfered with LFA-1-dependent leukocyte adhesion to ICAM-1. Consistently, Del-1 deficient endothelial cells promoted higher LFA-1-dependent leukocyte adhesion, as compared to wildtype endothelial cells under both static and physiologic flow conditions. Moreover, soluble Del-1 inhibited neutrophil recruitment in thioglycollate-induced peritonitis in vivo. As assessed by intravital microscopy in the dorsal skinfold chamber model, Del-1 deficiency enhanced slow rolling and firm adhesion of inflammatory cells. Furthermore, Del-1-/- mice displayed significantly higher neutrophil accumulation in LPS-induced lung inflammation in vivo and the pro-inflammatory phenotype of Del-1 deficiency was reversed in Del-1/LFA-1-double deficient mice. Thus, Del-1 is an endogenous inhibitor of inflammatory cell recruitment and could provide a basis for targeting leukocyte-endothelial interactions in disease.

2358/B65

Functional Analysis of Conserved Tyrosines in β Integrin Cytoplasmic Tail.

L. M. Hoang, J. Ahn, H. R. Wojciechowski, M. Lee; Biology, Baylor University, Waco, TX

Integrin is the αβ heterodimeric cell surface receptor for extracellular matrix (ECM) and an excellent model to investigate the function of conserved tyrosine (Y) phosphorylation motif, NPXY. The β integrin possesses two NPXY motifs, Y792 and Y804, in the cytoplasmic tail, and the phosphorylation of NPXY mediates interaction to a protein with phosphotyrosine binding (PTB) domain. Cell culture analyses suggested that Y to phenylalanine (F) mutation caused minimal defects while Y to alanine (A) abolished cellular function of β integrins. We characterized the activation of NPXY using βpat-3 integrin of the nematode Caenorhabditis elegans and generated a tyrosine (Y) to glutamate (E) mutant. βpat-3(Y804E), a transgenic mutant carrying a phosphomimetic mutation in the second NPXY motif of the βpat-3 cytoplasmic tail, showed defective muscles, abnormal gonad migration and tail morphology, ineffective mating, and high incidence in male. Some phenotypes of βpat-3(Y804E) paralleled those of him-4/hemicentin, an ECM molecule similar to human fibulin-5, leading us to hypothesize that defective him-4 causes phosphorylation in NPXY motifs of βpat-3 cytoplasmic tail. Thus, we introduced the βpat-3 non-phosphorylatable mutation, βpat-3(YYFF), in the him-4 background. Phenotypic analyses of βpat-3(YYFF); him-4 double mutant indicated that βpat-3(YYFF) mutation suppressed ineffective mating, high incidence in male, and egg-laying defects of him-4 males and hermaphrodites. This suggested that one of the functions of him-4/hemicentin is to prevent phosphorylation of βpat-3 NPXY. Further analysis will provide valuable information on the function of NPXY motifs in β integrin regulation and will enable us to interpret integrin signaling in other species.

2359/B66

Motifs in β5 Integrin Promoting RPE Cell-Specific Apical Polarity and Adhesive Function of αvβ5 Receptors.

M. Mallavarapu[1,2], S. C. Finnemann[2]; [1]Graduate Program in Physiology, Biophysics and Systems Biology, Weill Medical College of Cornell University, New York, NY, [2]Department of Biological Sciences, Fordham University, Bronx, NY

Retinal pigment epithelial (RPE) cells participate in two distinct cell-cell interactions in the eye. At all times, they maintain retinal integrity by adhering to photoreceptor outer segments and every morning, they phagocytose outer segment tips that shed in a circadian rhythm. Lack of the integrin receptor αvβ5 in β5 integrin null mice impairs both retinal adhesion and phagocytosis causing accumulation of lipofuscin in the RPE and blindness with age. Their essential role in both retinal adhesion and phagocytosis requires αvβ5 receptors to localize to the apical surface of RPE cells that faces photoreceptors. Indeed, αvβ5 is exclusively apical in the RPE in the eye. Its apical localization persists even in RPE cells in culture that lack contact with photoreceptors. Because other αv integrins localize basally in the RPE, we hypothesized that the cytoplasmic tail of the β5 integrin subunit contains motifs that promote the specific apical polarity of αvβ5 heterodimers. To identify such motifs, we generated vectors encoding full-length β5 integrin fused to GFP as well as β5-GFP mutants containing deletions or point mutations within the carboxiterminal 40 residues. Full-length β5-GFP was fully functional localizing apically in RPE and promoting phagocytosis and substrate adhesion. Notably, β5-GFP localized basally in polarized MDCK kidney epithelial cells suggesting that apical

polarity of αvβ5 integrin is RPE-specific. In non-polarized cells, all β5-GFP mutants reached the cell surface as αv heterodimers like full-length β5-GFP. However, deletion of β5 integrin regions 40-30, 30-20, or 20-10 residues from the C-terminus (but not 10-0) diminished αvβ5-GFP's ability to promote adhesion. We also mutated 7 single residues in the 30-20 region individually to alanine. Strikingly, all but one of these point mutations severely impaired adhesion. At present, we are comparing polarity of all our constructs expressed in RPE and MDCK kidney epithelial cells to pinpoint trafficking or retention motifs of β5 integrin specifically recognized by the RPE. These studies will improve our understanding of molecular mechanisms that control interactions between photoreceptors and the RPE.

2360/B67

The Role of Mac-1 (CD11b/CD18) in Osteoclast Differentiation Induced by Receptor Activator of Nuclear Factor-Kb Ligand (RANKL).

K. NAKAHAMA[1], H. Hayashi[1,2], T. Sato[3], I. Morita[1]; [1]Cellular Physiological Chemistry, Tokyo Medical and Dental University, Tokyo, Japan, [2]Toho University Omori Medical Center, Toho University School of Medicine, Tokyo, Japan, [3]Natural Resources and Physiological Chemistry, Tokyo Medical and Dental University, Tokyo, Japan

Objective: Osteoclasts are multinucleated bone-resorbing cells formed by the fusion of mononuclear cells derived from the monocyte/macrophage lineage (osteoclast precursors (pre-OCs)). Adhesion molecules are necessary for leukocyte trafficking and differentiation. It is well known that leukocyte function-associated antigen-1 (LFA-1, CD11a/CD18) plays important roles in osteoclastogenesis. This study examined the possible role of macrophage antigen 1 (Mac-1, CD11b/CD18), another integrin on pre-OCs, in osteoclastogenesis using RAW264.7 (macrophage cell line) cells and primary cultured bone marrow macrophages (BMMs). Methods: The RAW 264.7 cells were grown in α-MEM supplemented with 10% fetal calf serum. Sterile BMMs were obtained by flushing the femurs and tibias of ddY mice with phosphate-buffered saline. For knockdown of CD11b in RAW 264.7 cells, shRNA plasmid was transiently transfected into the cells using Lipofectamin 2000. Osteoclastogenesis was induced by RANKL (for RAW cells) or RANKL + M-CSF (for BMMs) in presence or absence of neutralizing antibodies against adhesion molecules. Osteoclast formation was evaluated using tartrate-resistant acid phosphatase staining. Quantification of nuclear factor of activated T cells c1 (NFATc1, a master regulator of osteoclastogenesis) mRNA was done by real-time RT-PCR. Results: RANKL-induced differentiation of RAW264.7 cells into multinuclear osteoclasts was dependent on the initial cell density. Fluorescence-activated cell-sorter sorting experiments showed impaired osteoclastogenesis in RAW264.7 cells with low CD11b expression. Neutralizing antibodies and siRNA against CD11b inhibited osteoclastogenesis induced by RANKL in RAW cells. Although BMMs expressed CD11a and CD11b, osteoclastogenesis induced by M-CSF and RANKL was inhibited in the presence of anti-CD11b or anti-CD18 but not anti-CD11a antibodies. Furthermore, anti-CD11b antibodies inhibited NFATc1 expression induced by M-CSF and RANKL in BMMs. Conclusions: Mac-1 may play an important role in the early stage of osteoclastogenesis via the communication between pre-OCs. Pre-OCs may recognize the existence of other pre-OCs to fuse with each other before the beginning of osteoclast differentiation.

2361/B68

Tetraspanin CD151 Mediates Adhesion-Dependent Activation of Ras, Rac, and Cdc42 by Facilitating the Formation of β1 Integrin-CD151-Small GTPase Complexes.

H. Lee[1], I. Hong[1], H. Byun[1], Y. Kim[2]; [1]Division of Life Sciences, Kangwon National University, Chunchon, South Korea, [2]Dept. of Biochemistry, School of Medicine, Kangwon National University, Chunchon, South Korea

We previously found that CD151 associates with α3β1 and α6β1 integrins and homophilic interactions of CD151 on two contacting cells provoke integrin-dependent c-Jun activation signaling pathways in human melanoma cells (JBC, 2006, 281:24279). In this study, we found that CD151 directly interacts with the small GTPases, Ras, Rac1, and Cdc42, but not Rho, through its cytoplasmic C-terminal region, leading to the formation of β1 integrin-CD151-GTPase complexes in the membrane. Homophilic CD151 interactions resulted in the activation of Ras, Rac1, and Cdc42 upon engagement of laminin-binding β1 integrins. CD151-associated β1 integrins appeared to up-regulate CD151-mediated activation of small GTPases not only by facilitating the association between CD151 and small GTPases but also by provoking FAK/Src-mediated pathway cross-talk. Ras, Rac1, and Cdc42 were found to transduce β1 integrin-CD151 adhesion receptor complex signals to p38 MAPK, JNK, and c-Jun in human melanoma cells, leading to increased cell motility and MMP-9 expression. Additionally, Ras appeared to act upstream of Rac1 and Cdc42 in β1 integrin-CD151 complex adhesion signaling pathways. These results suggest that CD151 functions as an adaptor, linking α3β1/α6β1 integrins to Ras, Rac1, and Cdc42, leading to adhesion-dependent activation of small GTPases.

2362/B69

Inactivation of Integrin-Linked Kinase in the Epidermis.

K. Nakrieko, S. J. D'Souza, L. Dagnino; Physiology & Pharmacology, University of Western Ontario, London, ON, Canada

Integrin-linked kinase (ILK) is a key contributor to cell proliferation, migration and adhesion. ILK exerts these effects by modulating signals between the cell and the extracellular matrix. To assess the role of ILK in epidermis, we generated mice with conditional inactivation of the *Ilk* gene in basal keratinocytes (hereafter termed K14Cre-ILK$^{f/f}$). This mutation causes perinatal lethality. ILK-deficient epidermis develops microblisters and appears to have abnormal adhesion to the basement membrane. The latter is associated with abnormal expression of integrin α6β4 in basal keratinocytes. In addition to these abnormalities in the interfollicular epidermis, hair follicles develop abnormally in K14Cre-ILK$^{f/f}$ mice. Specifically, there is a substantial reduction in the number of hair follicles, and those few follicles that form do not progress past the initial stages of development. In culture, primary ILK-deficient keratinocytes show defects in adhesion and migration, key events in hair follicle morphogenesis. To further examine the role of ILK in the hair follicle, we generated mice to conditionally inactivate the *Ilk* gene in epidermal stem cells of the hair follicle bulge (hereafter termed K15Cre-ILK$^{f/f}$). To study the role of ILK in hair follicle cycles, ILK gene expression was inactivated at various stages, including the first anagen at 10-15 days of age (P10-15), the second anagen (P30-35), and the first and second telogen (P20-25 and P49-54, respectively). The hair follicles in which *Ilk* had been inactivated showed normal transit through subsequent anagen and telogen phases with no obvious alterations in pelage phenotype. Immunohistochemical

analyses indicated no contribution of ILK-deficient cells to these hair follicles, suggesting that ILK is indispensable for a normal hair follicle cycle. To investigate the contribution of ILK to the hair follicle keratinocyte pool during epidermal regeneration, K15Cre-ILK$^{f/f}$ mice were wounded during the second telogen phase. Inactivation of the *Ilk* gene in keratinocyte stem cells resulted in significant delays in epidermal regeneration after injury. Our studies highlight an essential role for ILK in keratinocyte stem cells and epidermal regeneration.

2363/B70

Single Nucleotide Polymorphism of Integrin Alpha V in a Korean Population Was Associated with Susceptibility to Chronic Hepatitis B and HCC.

S. Lee[1], M. Kim[1], J. Cheong[2], S. Cho[2], S. Yang[3], H. Kim[1], K. Kim[1], J. Kim[1], A. Oh[1], J. Pyun[1], K. Lee[1], H. Song[1], S. Won[1], K. Kwack[1]; [1]Medical Genomics Laboratory, Pochon CHA University, Seongnam, South Korea, [2]Department of Gastroenterology, Genomic Research Center for Gastroenterology, Ajou University School of Medicine, Suwon, South Korea, [3]Macrogen Inc., Seoul, South Korea

Objective: Integrins are cell surface receptors for extracellular matrix proteins that initiate signaling pathways that modulate proliferation, survival, invasion, or metastasis. Consequently, integrins are potential targets for the treatment of cancer. In the injured liver, integrins and collagens are expressed by activated hepatic stellate cells and the increased expression of these genes is a commonly observed histological abnormality in hepatitis B infection. Despite the importance of integrin alpha V (ITGAV) in chronic HBV infection and HBV-infected HCC, little is known about genetic variations in ITGAV that may affect susceptibility to chronic HBV infection and the development of HBV-infected HCC. Methods: In the present study, we investigated whether 13 ITGAV SNPs in Korean patients confer greater susceptibility to chronic HBV infection and HBV-infected HCC. Thirteen ITGAV SNPs in 111 cases of chronic HBV infection, 86 cases of HBV-infected HCC, and 107 cases of acute self-limited HBV infection were genotyped by using Illumina's Sentrix Array Matrics chip. Results: The ITGAV intron SNPs rs9333289 and rs11685758, the 3′ untranslated region SNP rs1839123, and haplotype 3 were associated with enhanced susceptibility to HBV-infected HCC (p=0.02~0.05), while the intron SNP rs2290083 was associated with both chronic infection and HBV-infected HCC (p=0.01~0.04). In addition, both rs2290083 and haplotype 1 were associated with the age at which chronic infection occurred, as determined by Cox relative hazard analysis (RH=1.39~1.62, P=0.04~0.01). Conclusions: ITGAV SNPs and haplotypes may be genetic factors that increase the susceptibility of Koreans to chronic HBV infection and HBV-infected HCC.

2364/B71

Bit-1 Localization Determines Function and Is PI3K Dependent.

M. Matter, G. S. Griffiths, J. Astern, K. Sakata; Cardiovascular Research, John A. Burns School of Medicine, Honolulu, HI

Cell survival signals are modulated by many different receptors including receptor tyrosine kinases and integrins. Integrins themselves can influence cell survival through more than one pathway. Apoptosis that results from a loss of integrin-mediated cell attachment to the extracellular matrix is called anoikis. We have shown that a protein which we called Bit-1 is an effector of anoikis upon placing cells in suspension in serum containing media. Our objective was to extend these studies to examine Bit-1 function when cells are attached to specific matrices and serum starved. We used immunocytochemistry to determine whether integrin mediated attachment to fibronectin or collagen IV alters Bit-1 localization. Biochemical approaches were utilized to determine if Bit-1 co-immunoprecipitated with other proteins. Functional significance was examined using siRNA knockdown of Bit-1 expression. Finally, we examined cell survival using an MTT assay that measures cell viability. In cells attached to fibronectin Bit-1 is localized to the mitochondria. In addition, Bit-1 co-immunoprecipitates with Bcl-2. In contrast, when cells are plated on collagen IV Bit-1 is in the cytoplasm. Bit-1 localization is PI3K-dependent and forced expression of active PI3K upregulates endogenous Bit-1 levels. In attached cells knockdown of endogenous Bit-1 by siRNA decreased cell survival in serum free conditions and re-expression of Bit-1 abrogated this effect. Finally, attached cells that expressed endogenous Bit-1 were more resistant to staurosporine induced apoptosis compared to siRNA knockdown counterparts. This is in agreement with results published by others that Bit-1 null cells attached to the extracellular matrix are more sensitive than wild type cells to this treatment. In light of these data we have refined our model of how Bit-1 functions. Bit-1 is predominantly cytoplasmic and pro-apoptotic when no integrin is bound and is targeted to the mitochondria and anti-apoptotic when specific integrins are ligated.

2365/B72

Opposing Roles of α3β1 and α6β4 Integrins in Prostate Cancer Metastatic Colonization.

A. Varzavand[1], J. Drake[2], R. Svensson[2], M. Henry[2], C. S. Stipp[1]; [1]Biology, University of Iowa, Iowa City, IA, [2]Molecular Physiology & Biophysics, University of Iowa, Iowa City, IA

Laminin-5 mediates stable anchorage of normal epithelia, but may also contribute to invasion and metastasis of epithelial carcinomas. The two major receptors for laminin-5 are integrins α3β1 and α6β4. To determine the roles of α3β1 and α6β4 in tumor cell response to laminin-5, we used RNA interference technology to efficiently silence α3, α6, or both α3 and α6 in PC3-Li cells, an aggressive, α6β4-positive subline of PC3 prostate carcinoma cells, isolated from a liver metastasis. Near total loss of α3 but not α6 severely impaired PC3-Li cell adhesion on laminin-5, but not on the α2β1 integrin ligand, collagen I. In survival assays in serum free medium, laminin-5 could compensate for the lack of serum growth factors, and this was strongly dependent on α3 but not α6 integrin. In motility assays on laminin-5, the α3-silenced cells did not adhere well enough for quantification; however, α6-silenced cells adhered and migrated with wild type velocity. The metastatic colonization potential of the different cell types was monitored by bioluminescence imaging in an experimental model of lung metastasis. In contrast to the in vitro results, which emphasized the role of α3β1, in the in vivo assay, α6-silenced cells displayed impaired colonization, while the loss of α3 actually appeared to enhance lung colonization. Results from the α3/α6 doubly-silenced cells suggest that α6 integrin's tumor promoting activity may at least partly derive from inhibiting α3 integrin's tumor suppressive activity in this model.

2366/B73

Heterodimeric Interaction of Integrin α and β Transmembrane Domains (TMD) Controls Integrin Signaling.

C. Kim[1], T. Lau[2], F. Ye[1], T. S. Ulmer[2], M. Ginsberg[1]; [1]Department of Medicine, University of California, San Diego, La Jolla, CA, [2]Department of Biochemistry and Molecular Biology and Zilkha Neurogenetic Institute, University of Southern California, Los Angeles, CA

Bidirectional integrin signaling controls the assembly of the extracellular matrix and cellular responses to the assembled matrix. Previous mutational and Cysteine-cross-linking studies suggested that the packing of the integrin α and β TMD regulated these signals; however biochemical studies in lipid micelles and genetic analyses in bacterial membranes failed to detect these interactions and instead indicated preferential homodimeric and homotrimeric association of integrin α and β TMD. Here we have combined a novel affinity capture approach with Nuclear Magnetic Resonance spectroscopy to, for the first time, detect the interaction of integrin αIIb and β3 TMD in mammalian cells and in small bicelle model membranes. Furthermore, we report that the recombinant αIIb TMD binds to and activates native integrin αIIbβ3; mutations in αIIb TMD region that disrupt its association with β3 TMD block the ability of the over-expressed TMD to activate αIIbβ3. Conversely, introduction of these mutations into native αIIbβ3 leads to constitutive integrin activation. Furthermore, early studies suggested that a salt bridge between αIIb(R995) and β3(D723) regulates integrin signaling. We now provide proof of that this salt bridge is important in stabilizing the association of integrin α and β TMD in mammalian cell membranes and in model lipid bicelles. Finally, talin binding to integrin β cytoplasmic domains leads to integrin activation in cells; we now find that talin binding activates highly purified αIIbβ3 in model membranes and that talin binding inhibits the αβ TMD interaction. Thus we provide direct evidence that preferential integrin αβ TMD interaction occurs and regulates integrin signaling in mammalian cells, that a salt bridge between the highly conserved β3(723) and αIIb(R995) stabilizes this interaction, and that talin binding to the β3 cytoplasmic domain disrupts it.

2367/B74

Cell Adhesion to Thrombin Peptide, TP508, Is RGD-Dependent and Mediated by Integrin αvβ3.

D. N. Derkach[1], S. A. Wadekar[1], E. Rousseau[1], T. Ugarova[2,1], K. Perkins[1], M. Sheller[1]; [1]R&D, OrthoLogic Corp., Tempe, AZ, [2]School of Life Sciences, Arizona State University, Tempe, AZ

TP508 is a 23-amino acid synthetic peptide that represents residues 508 to 530 of human prothrombin and elicits a variety of potential therapeutic effects such as acceleration of wound healing, revascularization and bone fracture repair. Despite the discovery of a wide range of effects associated with TP508 treatment, the identity of a TP508-receptor has remained elusive. TP508 contains a classic RGD integrin binding motif that comprises residues 517 to 519 of human prothrombin and can potentially associate with integrins. The objective of this study is to determine whether TP508 supports integrin-mediated cell adhesion. These studies show that immobilized TP508 supports efficient adhesion of human aortic endothelial cells (HAE) and integrin $\alpha_v\beta_3$-expressing HEK293 cells. Adhesion was concentration-dependent with maximal adhesion being greater than 50% of that observed on immobilized fibrinogen. Furthermore, adhesion was RGD-dependent, as scrambled TP508 or mutant TP508 in which Gly in the RGD motif was substituted to Ala (TP508-RAD) did not support adhesion. Soluble TP508 can also inhibit cell adhesion to immobilized fibrinogen in a dose-dependent manner. This inhibition is RGD-dependent as TP508-RAD and scrambled TP508 did not significantly interfere with cell adhesion to fibrinogen. The integrin $\alpha_v\beta_3$ was implicated in recognition of TP508 as anti-$\alpha_v\beta_3$ function-blocking antibody LM609 and anti-β_3 specific mAb 7E3 significantly reduced the number of adherent cells. In addition, adhesion to TP508 appears to be dimer-dependent as S-TP508 (Cys521□Ser, a mutant of TP508 that does not form dimers) exhibits significantly reduced cellular adhesion. These results identify TP508 as a new ligand for integrin $\alpha_v\beta_3$ and demonstrate that the interaction of TP508 with $\alpha_v\beta_3$ is RGD-dependent.

2368/B75

Inter-Spatial Occupancy of ERM Proteins between Two Adjacent Intracellular Domains of ICAM-1 Determines Cell Surface Dimerization.

H. Oh, H. Choi, H. Kim, C. Jun; life science department, Gwangju institute science and technology, Gwangju, South Korea

Dimeric intercellular adhesion molecule-1 (ICAM-1) has been known to more efficiently mediate cell adhesion than monomeric ICAM-1. Here, we found that truncation of cytoplasmic domain of ICAM-1 significantly enhances surface dimerization based on the two criteria; 1) the binding degree of monomer specific antibody CA-7 and 2) the ratio of dimer/monomer when mutation (L42→C42) was introduced in the interface of domain 1. The C-terminal 12 amino acids were dispensable, whereas the remaining residues including a segment of 5 amino acids 507RKIKK511 were required for the equilibrium state between dimer and monomer. Surprisingly, targeted knock down of ezrin and moesin significantly increased dimerization in wild-type ICAM-1, suggesting that inter-spatial occupancy of ERM proteins between two adjacent cytoplasmic domains is essential. Despite a strong dimer presentation, ICAM-1 mutant lacking cytoplasmic domain supported lower degree of cell adhesion than wild-type ICAM-1 and revealed an impaired trafficking to the immunological synapse. Overall, these results demonstrate that the effectiveness of ICAM-1 is dependent on, not the dimeric state, but the dynamic flexibility of monomer-dimer transition. Notably, this is the first evidence, at least in the immunoglobulin superfamily member, that architecture of extracellular domain is determined by the intracellular domain and associated proteins.

2369/B76

Use of Function Blocking Antibodies to Identify the Specific Integrin Subunits Involved in Bovine Fertilization.

J. M. Brinkerhoff, B. R. Sessions, K. L. White; Animal Dairy and Veterinary Sciences, Utah State University, Logan, UT

Integrins are heterodimers composed of two subunits, alpha (α) and beta (β), and facilitate cell migration and attachment to the extra-cellular matrix, mediate cell-cell adhesion, and act as two-way signaling molecules. Integrins located on the vitelline membrane of bovine oocytes have been implicated in mediating the sperm-oocyte interaction (Campbell et al., 2000; Sessions et al., 2006) and the identity of the specific integrin subunits present on the bovine oocyte has also been determined (αV, α2, α4, α6, β1, and β3; Pate et al., 2006). Anti- integrin function blocking antibodies were utilized in order to reveal which specific integrin subunits are involved in mediating the sperm-oocyte interaction during bovine fertilization. Oocytes were pre-incubated with function blocking antibodies specific for the integrin subunits located on the vitelline membrane of bovine oocytes and subsequently fertilized and cultured in vitro according to our standard laboratory procedures (Reed et al., 1996). The oocytes pre-incubated with the function blocking antibodies for the αV and β1 subunits had significantly lower cleavage

rates (p <.05) compared to all other treatment groups. These data are compelling evidence that the αV and β1 integrin subunits are essential for fertilization in the bovine and could form the integrin heterodimer involved in the sperm-oocyte interaction.

2370/B77

Interaction of Integrin Beta4 with FAK in Promoting Tumor Progression.

Y. Tai[1], H. Tseng[1], M. Abdel-Ghany[2], I. Lai[1], B. Pauli[2], J. Guan[2], T. Shen[1]; [1]Department of Plant Pathology and Microbiology, National Taiwan University, Taipei, Taiwan, [2]Department of Molecular Medicine, Cornell University, Ithaca, NY

Both integrin β4 and focal adhesion kinase (FAK) play an important role in the tumor progression and FAK is a component in transducing signals downstream of integrin β4. Here, we demonstrated a physical and functional association between these two molecular in vivo and in vitro. An eleven amino-acid motif within the amino-terminus of FAK was identified to be responsible for interacting with the cytoplasmic tail of integrin β4. Furthermore, two of the eleven amino acids within the amino-terminus of FAK acted as crucial positions for binding to integrin β4. Furthermore, the interaction is an adhesion-dependent manner and, subsequently, resulted in the phosphorylation and activation of FAK in both HCT116 and MDA-MB-231 cells. In addition, EGF signaling and the activity of Src also contributed to this interaction, whereas PI3 kinase was indispensable. Interestingly, this interaction was not observed in MCF7 cells, implicating that the involvement of this interaction in tumor progression. In addition, in HCT116 and MDA-MB-231 cells, disruption of the interaction between integrin β4 and FAK reduced cell proliferation and motility, suggesting the requirement of this interaction in tumorigenesis and perhaps metastasis in these tumor cells. Our data resolved for the first time a novel pathway of integrin β4-mediated cellular function via direct interaction with FAK to regulate tumor progression, thereby strengthening a link between integrin β4 and FAK biochemically and functionally. Key words: integrin beta4, FAK, tumorigenesis, metastasis

Gap Junctions II (2370 – 2398)

2371/B78

Gap Junction as a Critical Component of the Blood-Testis Barrier in Testes.

W. Li[2], C. Cheng[1]; [1]Population Council, Center for Biomedical Research, New York, NY, [2]School of Biological Sciences, The University of Hong Kong, Hong Kong, China

Formed by adjacent Sertoli cells in the testis, the blood-testis barrier (BTB) creates a unique microenvironment in the seminiferous epithelium for post-meiotic germ cell development. The BTB is composed of co-existing tight junction (TJ), basal ectoplasmic specialization (ES), desmosome-like junction and gap junction (GJ). Its regulation, however, remains largely unknown. The expression of connexin 43 (Cx 43), the predominant GJ protein in testes, is stage-specific. It was predominantly expressed at the BTB. Its expression diminished from stage VIII to an almost undetectable level at stages IX-X, coinciding with the BTB restructuring which facilitates the transit of preleptotene spermatocytes. Cx 43 was also detected at the apical ES at the Sertoli cell-elongating spermatid interface but its staining diminished to an undetectable level at late stage VIII at spermiation. Thus, GJ likely serves as a signal transducer among different junction types at the BTB to coordinate its restructuring during the epithelial cycle. When adult rats (n = 3) were fed with a single dose of adjudin (50 mg/kg b.w.) to induce spermatid depletion via anchoring junction restructuring, Cx 43 staining at the apical ES was virtually undetectable in the departing spermatids. However, the intensity of its staining at the BTB increased significantly. Collectively, these data illustrate that Cx43 may participate in the maintenance of the cell adhesion at the apical ES and BTB. To further delineate the role of GJ in the BTB function, Cx 43 was knockdown by specific Cx 43 siRNA duplexes in Sertoli cell cultures with an established TJ-permeability barrier that mimicked the BTB *In Vivo*. When Cx43 was knockdown by RNAi by ~50%, immunoblot analysis illustrates that the levels of TJ (occludin, JAM-A, or ZO-1), basal ES (N-cadherin, or catenins), GJ (Cx26), desmosome-like junction (desmoglein-2), and signal transducers (c-Src, ERK1/2, or p-ERK1/2) remained unaltered. However, an apparent mislocalization of occludin, but not ZO-1 or β-catenin, was detected in Sertoli cells by fluorescent microscopy. In summary, GJ and Cx 43 could be important for the maintenance of the BTB function.

2373/B80

Establishing a Rat Mast Cell Line for the Study of Gap Junctional Intercellular Communications.

T. T. Foley, G. C. Saggers, H. Ehrlich; Plastic Surgery, Penn State Hershey Medical Center, Hershey, PA

Gap Junctional Intercellular Communications (GJIC) occur between HMC-1, a human mast cell (MC) line, and human fibroblasts cast in co-cultured fibroblast populated collagen lattices (FPCL), which increases the rate and degree of lattice contraction. In contrast, GJIC between HMC-1 and fibroblasts do not occur in monolayer culture. Freshly isolated rat peritoneal MC, which have a short survival time, institute GJIC with fibroblasts in monolayer. To further study GJIC between MC and fibroblasts, the establishment of a primary MC line is needed. The HMC-1 cell line does not require cytokine supplementation for preservation, while other cultured MC do. The hypothesis is HMC-1's release cytokines that promote the proliferation of isolated MC. MC isolated from rat peritoneal lavages were incubated with spent HMC-1 media at a ratio of 7:3 to DMEM with only bovine serum. Immuno-histology demonstrated the establishment of rat MC cell lines at 2 weeks. Cultured rat MC proliferated without supplements. MC paratroopers were created to demonstrate GJIC between MC and fibroblasts. MC plasma membranes were stained fluorescent red and a green fluorescent dye, was trapped within their cytoplasm. MC paratroopers passed green dye into fibroblasts, which were distinguished from MC paratroopers by having only green fluorescence. MC paratroopers pretreated with a synthetic fatty acid amide hydrolase (FAAH) inhibitor that blocks the breakdown of oleamide, which disrupts GJIC, did not pass dye. To demonstrate enhanced co-cultured rat MC-FPCL contraction, MC and fibroblasts were cast together in collagen lattices. At 48 hrs FPCL area was 37 ± 7 mm2 and the MC-FPCL area was 29 ± 4 mm2 (p > 0.001). When FAAH inhibitor pretreated MC were incorporated in lattices, no enhanced lattice contraction occurred. The establishment of a rat MC line that creates GJIC with fibroblasts facilitates the study of GJIC between MC and fibroblasts in fibrosis and scarring.

2374/B81

Gap Junction Communication and Apoptosis by in Phases of the Cell Cycle of GFSHR-17 Granulosa Cells.

S. Schlie[1], K. Mazur[1], B. Chichkov[2], A. Ngezahayo[1]; [1]Institute of Biophysics, Leibniz University Hannover, Hannover, Germany, [2]Laserzentrum e.V., Hannover, Germany

Previous results have shown that the inhibition of gap junction coupling in GFSHR-17 granulosa cells correlated with induction of apoptotic reactions such as chromatin condensation, DNA strand break and formation of blebs. In the present report the double whole-cell patch-clamp technique on synchronised cells revealed that the macroscopic conductance of gap junctions in mitotic phase was significantly reduced in comparison to the cells in interphase. Additionally, the inhibitor of guanylyl cyclase ODQ which was shown to inhibit the gap junction coupling and promoted activation of characteristic apoptotic reactions in GFSHR-17 granulosa cells. In synchronised cells we found that the induction of both chromatin condensation as well as DNA strand break by ODQ was significantly increased in cells in mitotic phase compared to cells in interphase. These effects of ODQ were not observed in HeLa cells which do not express connexins. The results suggest a more sensitivity to stimulation of the apoptotic reactions by ODQ linked to a reduced gap junction coupling in cells in mitotic phase.

2375/B82

Stat3 Inhibition Eliminates Gap Junctional Communication in Lung Carcinoma Cells.

C. Chaize[1], M. Geletu[1], R. Arulanandam[2], K. Firth[1], L. Raptis[1,2]; [1]Microbiology and Immunology, Queen's University, Kingston, ON, Canada, [2]Pathology and Molecular Medicine, Queen's University, Kingston, ON, Canada

Gap junctions are plasma membrane channels connecting the cytoplasm of adjoining cells. A large number of oncogenes have shown to eliminate gap junctional, intercellular communication (GJIC). Previous results indicated that the vSrc oncogene is able to interrupt GJIC by a mechanism which requires the action of Ras. Since the vSrc oncogene also activates the Signal Transducer and Activator of Transcription-3 (Stat3), we examined the effect of Stat3 inhibition upon GJIC in A549 lung carcinoma cells which have high Src activity levels and, as expected, gap junctional communication is undetectable. GJIC was examined using a novel technique of *in situ* electroporation: Cells are grown directly on two transparent thin film electrodes that partially cover a glass slide. A wall bonded to the slide creates a chamber to contain growth medium and cells. A barrier divides the chamber in half such that, on each side, there is an electrode with cells growing on it and an adjacent region of cells that are growing on non-conductive glass. A tracking dye (Lucifer Yellow, LY) is added to the medium. An electrical pulse is applied through the electrodes which are connected via the growth medium that fills the chamber beyond the height of the dividing barrier. The pulse causes transient pores to form in the cells growing on the electrodes so that LY penetrates. The subsequent migration of the dye to the non-electroporated cells on the non-electrode (bare glass) part of the slide is microscopically observed under fluorescence illumination. The results revealed no increase in GJIC upon Stat3 inhibition with a platinum compound, a dominant-negative mutant or shRNA, indicating that, unlike cRas, Stat3 does not participate in gap junction closure in this system. To further examine the effect of Stat3, we examined GJIC levels in the QUDB, lung carcinoma line, which has extensive junctional permeability. Unexpectedly, Stat3 inhibition caused a dramatic reduction in GJIC, concomitant with apoptosis induction. These results show that Stat3, while able to promote cell division and transform cultured cells in an activated form, is also able to *promote* junctional communication, a finding which could have important therapeutic implications.

2376/B83

DNA Damage in Human Cells after Indirect Exposure to CoCr Nano-Particles through a Placental Cell Barrier.

A. Sood, G. Bhabra, C. Case; UoB, Bristol, United Kingdom

Humans are exposed to nano-sized chromium particles from cobalt chrome orthopaedic joint replacements. In previous studies we have shown that human fibroblasts show DNA and cytogenetic damage in tissue culture after exposure to CoCr particles and ions. In this novel study we have asked the question whether human cells might also show DNA damage if separated from the metal by a placental cell barrier. We created a barrier 3-5 cells in thickness of BeWo cells (as a standard model of a placental barrier) and exposed the barrier to nanometer (29nm) and micron (3.4µm) sized CoCr particles, as well as ions of Co2+ and Cr6+ individually or in combination. We monitored DNA damage in BJ fibroblasts beneath the barrier with the alkaline gel electrophoresis comet assay and with γH2AX staining. The results showed evidence of DNA damage after all types of exposure. The indirect damage (through the barrier) was equal to the direct damage at the concentrations tested. The integrity of the barriers was checked with measurements of electrical resistance (TEER values) and permeability to sodium fluorescein (376Da) and found to be intact. In light of these results and with the knowledge that BeWo cells express cx43, we tested the theory that a damaging signal was being relayed via gap junctions or hemi channels in the BeWo cells to the underlying fibroblasts. We used the connexin mimetic peptides Gap19 and Gap26 (known to selectively block hemichannels and gap junctions respectively) and 18α-glycyrrhetinic acid (non-selective gap junction blocker). All of these compounds completely obliterated the indirect damaging effect seen in our previous experiments. We conclude that CoCr particles can cause DNA damage through a seemingly intact barrier, and that this damage occurs via a bystander mechanism. It would be of interest to test whether other barriers (e.g pleura, peritoneal) are 'leaky' to DNA damaging effects of nanoparticles and whether this is simply a tissue culture effect or could be seen in vivo.

2377/B84

Role of Gap Junctions in Mammalian Oogenesis; Connexin Genes Expressed in Follicle-Like Structures Derived from Mouse Embryonic Stem Cells.

M. Nitta[1,2], J. Miyoshi[2], T. Takeya[1]; [1]Nara Institute of Science and Technology, Ikoma, Japan, [2]Research Institute, Osaka Medical Center for Cancer and Cardiovascular Diseases, Osaka, Japan

Mammalian oogenesis proceeds in ovarian follicles through the interplay between oocyte and its surrounding follicle cells. Gap junctions play a crucial role for this communication by transporting small molecules such as metabolites, ions and second messengers, which are required for the growth of both oocytes and follicle cells. In mouse ovary, several connexins are reported to be expressed in stage- and region-specific manners; for example connexin 43 (Cx-43) expresses in granulosa cells throughout the folliculogenesis, whereas Cx-37 in the oocyte. Deletion of either gene is known to abrogate normal folliculogenesis and oogenesis, respectively. To understand the gap junctional

communication in ovarian follicles at the molecular level, we are trying to establish an in vitro culture system that enables us to observe the formation of mature ovum from mouse ES cells, so that we can perform the functional analysis of connexins in a spatiotemporal manner. To this end, we first defined experimental conditions to obtain follicle-like cell complexes starting from several strains of mouse ES cells. We consequently observed the formation of cell complexes showing follicle-like structure comprising of oocyte-like cells surrounded by several layers of follicle-like cells. Analysis of serial section of these complexes actually revealed the presence of oocytes in the center with zona pellucida and basal lamina, in addition to one or several surrounding layers of granulosa cells. Furthermore, expression of several genes known to be unique to germ cells could be detected, confirming that these follicles contained germ cells and have entered into meiotic stage. Genes encoding kit-ligand and Cx-37, known to be essential for oogenesis, were also expressed. No follicular antrum, however, were observed, suggesting that the derived follicles seemed to be arrested at the stage of primary or secondary follicles. Finally, we observed the expression of Cx-43 in the region between the oocyte and follicle cells as well as between follicle cells themselves in mouse ovary as has been reported, and this expression profile could be observed by immunostaining in induced follicle-like cell complexes.

2378/B85

Tryptophan-Substitution Experiments Reveal Putative Sites of Transmembrane Domain Interaction in Cx32.

J. Karcz, I. M. Skerrett; Biology, Buffalo State College, Buffalo, NY

A tryptophan-scanning technique was applied to the first transmembrane domain (M1) of connexin32 (Cx32) with the aim of identifying sites that interact closely with other transmembrane helices. The technique has been used successfully to identify sites of transmembrane domain interaction in other proteins and is based on the premise that the large bulky side-chain of tryptophan is tolerated when positioned in a lipid environment but disrupts protein function when inserted at a site of protein interaction. Tryptophan was substituted sequentially for amino acids within M1 of Cx32 and channel function was assayed using the Xenopus oocyte expression system. At several sites toward the cytoplasmic end of M1, tryptophan substitution failed to to alter channel expression or function. In contrast, substitution at three sequential sites predicted to lie in the middle of M1 completely abolished intercellular coupling suggesting that this region interacts closely with other transmembrane helices.

2379/B86

Cx46, a Hypoxia Specific Gap Junction Which Protect Tumors from Hypoxia.

D. Banerjee[1], D. Madgwick[1], V. Akoyev[1], G. Gakhar[2], D. Takemoto[1], A. Nguyen[2]; [1]Biochemistry, Kansas State University, Manhattan, KS, [2]Diagnostic Medicine/Pathobiology, Kansas State University, Manhattan, KS

Objective: Normal tissues do not survive under hypoxia. The exceptions are lens of the eye (naturally hypoxic) and solid tumors (adaptive hypoxic) prior to vascularization. Connexin 46 (Cx46) is a novel gap junction protein that appears during lens fiber formation, inside the hypoxic center of the lens. We have found solid tumor such as breast tumor also express Cx46.The purpose of the study is to determine if Cx46 protein protects solid tumors, such as breast tumors, and lens from hypoxia. Method: Expression of Cx46 in solid breast tumor tissue, several breast tumor cell lines and lens cell lines was verified by immunoblot and immunohistochemistry. To verify that Cx46 protects cells from hypoxia ,(a) neuronal N2A cells over-expressing Cx46 (b) human lens epithelial cells(HLEC), and (c) breast cancer cell line MCF-7 ,with Cx46 downregulated by siRNA, were incubated under hypoxic condition (1% O2) and cell viability was assessed .To determine if down-regulation of Cx46 reduces breast cancer tumors in vivo , a tumor xenograft model was used where mice were inoculated with estradiol-17B and injected with 107 breast cancer to develop breast tumor. Mice with tumor xenografts were injected with Cx46 siRNA, in every 48 hours, and tumor size was measured up to 10 days with multiple siRNA injection. Result: The Cx46 was upregulated in response to hypoxia in lens epithelial NN 1003A cells. Breast cancer cell lines which had elevated estrogen receptor also had a high level of Cx46. N2A cells with endogenous Cx46 were more resistant to death caused by hypoxia than N2A cells with no Cx46.The downregulation of Cx46 by siRNA in HLEC and MCF-7 cell line made them vulnerable to hypoxia induced cell death. In vivo studies showed that the tumor growth was arrested in mice injected with siRNA against Cx46. Conclusion: Cx46 plays an important role to protect breast tumor and lens from hypoxia. The above work is innovative and important as it is the first to compare a naturally hypoxic tissue, the lens, with a diseased hypoxic tissue, breast tumor, for their survival strategies involving a novel gap junction protein connexin 46. Results clearly demonstrate a novel role for Cx46 in protection from hypoxia.

2380/B87

Differential Permeability of Molecules through Gap Junctions Formed by Connexins Expressed in the Lens.

Y. Shen[1], X. Li[2], A. P. Moreno[3], G. S. Goldberg[1]; [1]Molecular Biology, UMDNJ-SOM, Stratford, NJ, [2]Molecular Biology and GSBS, UMDNJ-SOM, Stratford, NJ, [3]Nora Eccles Harrison Cardiovascular Research and Training Institute, University of Utah, Salt Lake City, UT

Gap junctions are composed of connexins which form aqueous channels between cells. Gap junctional communication is important for homeostasis and development of many types of tissues and organs. For example, Cx43, Cx46, and Cx50 are expressed in the lens, and Cx46 knockout mice develop nuclear cataracts, while Cx50 knockout mice exhibit microphthalmia and zonular pulverulent cataracts. Mutations in Cx46 and Cx50 are also associated with cataracts in humans. However, Cx43 can not substitute for Cx46 or Cx50 to prevent cataract formation. Therefore, selective transfer of specific molecules through channels formed by Cx43, Cx46, or Cx50 may be required for normal lens development and function. We have created a cell system to investigate differential permeability of channels formed by these connexins. For example, while small ions can pass between cells through all of these channels, some larger charged molecules transfer preferentially through channels formed by Cx43.

2381/B88

Modulation of Cell-Cell Electrical and Metabolic Communication by Transjunctional Voltage in Heterotypic Gap Junction Channels.

N. Palacios-Prado, F. Bukauskas; Neuroscience, Albert Einstein College of Medicine, Bronx, NY

Heterotypic gap junction (GJ) channels form between cell expressing different connexin (Cx) isotypes. Our earlier reported and unpublished data show that heterotypic GJs in which Cx45 hemichannel is docked with Cx31, Cx40, Cx43, Cx47 and assumingly other Cx isotypes exhibit strong voltage gating asymmetry and that cell-to-cell electrical signaling can be modulated from bidirectional to unidirectional. Here we show that dye transfer between cells expressing these heterotypic GJ channels can be efficiently modulated by relatively small transjunctional voltage (Vj) or electrical activity of high frequency applied to different sides of heterotypic junctions. Studies were performed in homologous and heterologous cell pairs of HeLa cells expressing Cx43-EGFP and Cx45-CFP that form Cx43-EGFP/Cx45-CFP heterotypic GJ channels. We show that permeability of Cx43-EGFP/Cx45-CFP junctions for fluorescent dye molecules, such as Alexa fluor-350 and Lucifer Yellow can be efficiently blocked or enhanced by making Cx45-CFP side relatively negative or positive, respectively. Dye transfer was noticeably reduced when voltage steps that are similar in amplitude and duration to action potentials (~+100 mV and 3-50 ms, respectively) are applied with high frequency (~10-200 Hz) to the cell expressing Cx43-EGFP, and enhanced when applied to the cell expressing Cx45-CFP. Modulation of permeability by Vj in heterotypic GJs may be a new and important way to regulate the metabolic intercellular communication and cell-to-cell signaling in tissues where Cx45 is most abundantly co-expressed with other connexins, such as the conduction system of the heart, blood vessels and nervous system.

2382/B89

Atrial Fibrillation-Associated Connexin40 Mutations Alter Electrophysiological Properties of Gap Junction Channels.

J. Gemel[1], X. Lin[2], R. Veenstra[2], E. Beyer[2]; [1]Pediatrics, University of Chicago, Chicago, IL, [2]Department of Pharmacology, SUNY Upstate Medical University, Syracuse, NY

Atrial fibrillation (AF) is the most common arrhythmia affecting at least 6% of people over 65 years old. Recently, it has been suggested that mutations in human connexin40 (hCx40) may predispose the atria to fibrillation. Both somatic (P88S, G38D, and M163V) and germline (A96S) mutations of Cx40 were identified in patients with AF by Gollob et al. (N.Engl.J.Med. 354:2677-88, 2006). This study was undertaken in order to characterize further these AF-related Cx40 mutations. Communication-deficient HeLa and N2a cells were transfected with plasmids containing each of the mutants studied by immunochemistry and dual whole cell patch clamp techniques. Immunoblotting confirmed production of the introduced mutant proteins. Immunofluorescence showed that P88S localized within the cytoplasm, but each of the other mutants localized to appositional membranes. P88S did not induce junctional currents. G38D, M163V and A96S each produced intercellular currents, but the properties of their channels differed from those of wild type Cx40. G38D exhibited a reduced half inactivation voltage ($V_{1/2}$) and an increased unitary channel conductance (γ_j), while M163V had an increased $V_{1/2}$ and reduced γ_j. A96S did not change $V_{1/2}$ or γ_j, but it did still produce a reduction of macroscopic gap junctional conductance. These changes in the behavior of hCx40 mutant channels may contribute to the modification of electrical impulses propagation in human heart when expressed alone or together with other heart connexins.

2383/B90

The Increase in Connexins 39, 43 and 45 Levels in Isolated Rat Skeletal Myofibers Without Contractile Activity Is Prevented by IL-6.

L. A. Cea[1], M. A. Riquelme[1], J. von Maltzahn[2], K. Willecke[2], J. C. Sáez[1]; [1]Department of Physiology, Pontificia Universidad Católica de Chile, Santiago, Chile, [2]Institut für Genetik, Universität Bonn, Bonn, Germany

Connexins (Cxs), gap junction channel and hemichannel protein subunits are not present in adult skeletal muscles. However, during myogenesis or regeneration, Cxs 39, 43 and 45 are detected transitorily. Adult skeletal muscles release different autocrine/paracrine factors under resting condition. One of them is interleukin-6 (IL-6), which is released preferentially during exercise. IL-6 reduces the Cx32 levels in a hepatocyte cell line and prevents the formation of gap junctions between activated dendritics cells. The aims of this work were: 1) to study if Cxs 39, 43 and 45 increase in isolated skeletal myofibers without contractile activity; 2) if IL-6 prevents the increase in Cx levels, and 3) if these Cxs form functional hemichannels (HCs). Myofibers were isolated from *flexor digitorium brevis* muscle by collagenase digestion for 3h at 37°C. Relative Cx levels were evaluated by immunoblotting and immunofluorescence. The presence of functional Cx HCs was evaluated through the ethidium (Etd) uptake sensitive to Cx HC blockers in time lapse measurements under basal conditions and after electrical stimulation. Levels of all Cxs increased progressively over time in cultured skeletal myofibers, with maximal response at 48h of culture. Treatment with IL-6 (50ng/ml) for 24h prevented the increase in Cx levels. Freshly isolated myofibers (4h culture) presented functional HCs under basal condition and electrical stimulation increased their activity □4-fold. Whereas, in myofibers cultured for 48h the electrical stimulation increased the HC activity □6-fold. At both times the Etd uptake was blocked with 18β-glycyrrhetinic acid. In myofibers cultured for 48h treatment with IL-6 during the last 24h reduced the electrical stimulation elicited Etd uptake. Therefore, in adult skeletal muscle, down regulation of Cxs 39, 43 and 45 is controlled by IL-6.

2384/B91

Characterization of Pannexin1 and Pannexin3 Trafficking and Cell Surface Dynamics.

R. Bhalla, S. Penuela, J. Simek, Q. C. Shao, D. W. Laird; Anatomy and Cell Biology, University of Western Ontario, London, ON, Canada

Being mammalian orthologs of the invertebrate gap junction proteins innexins, pannexins have been proposed to exhibit similar cellular characteristics to connexins. To assess the trafficking and secretory pathways utilized by pannexins and to directly compare these events with the most characterized connexin family member, Cx43, we utilized Cx43-positive BICR-M1Rk cells to generate stable cell lines co-expressing Panx1, Panx3 or Panx1-GFP. Panx1 and Panx3 were found to be capable of trafficking to the cell surface; however, their distribution profile typically reflected a uniform pattern while Cx43 localized into punctuate structures at cell-cell interfaces. By expressing a dominant negative mutant of Sar1 GTPase, we found that similar to Cx43, Panx1 and Panx3 were retained within an intracellular compartment suggesting that they share a COPII-dependent secretory pathway. Western blotting further supported an ER to Golgi trafficking pathway of Panx1 and Panx3, as the Gly0 and Gly1 species of Panx1 and the lower molecular weight species (~43kD) of Panx3, accumulated in the presence of both Sar1-mutant and long term BFA treatment. Interestingly, microfilament disruption with cytochalasin B caused a notable redistribution of Panx1-GFP to the intracellular compartments indicating the role of microfilaments in Panx1 cell surface stability.

Conversely, the cell surface population of Panx1-GFP appeared insensitive to nocodazole treatment while Cx43 trafficking and distribution is facilitated by intact microtubules. Fluorescent recovery after photo-bleaching revealed that Panx1-GFP was considerably more mobile at the cell surface in comparison to Cx43-GFP localized to gap junction plaques, suggesting that Panx1-GFP reaches a substantially different state of assembly or clustering. Collectively, these studies show that even though Panx1 and Panx3 share a common ER to Golgi secretory pathway with Cx43, they acquire distinct distributions and dynamic states at the cell surface reflecting the unique properties of these molecules. Work supported by CIHR to DWL and NSERC to RB.

2385/B92

Growth Suppression by Cx37 Requires a Functional Pore Domain.

M. E. Good, T. K. Nelson, J. M. Burt; Physiology, University of Arizona, Tucson, AZ

Connexin (Cx) 37, unlike Cx40 or Cx43, suppresses the proliferation of Rat insulinoma (Rin) cells by, as yet, unknown mechanisms but possibly related to unique properties of the channels formed (gap junction or hemichannel) or unique protein-protein interactions. We hypothesized that a functional pore domain was not necessary for Cx37's anti-proliferative effects. To test this hypothesis, we introduced a mutation that converted threonine 154 to alanine (T154A). Mutation at this site in other alpha and beta connexins results in a channel dead protein that otherwise preserves most, if not all, other functions of the connexin. Rin cells were stably transfected with Cx37-T154A (under the tetracycline inducible promoter) and multiple clonal populations that demonstrate doxycyline dose-dependent expression of the protein were isolated. In these clones, the expressed protein localizes comparably to Cx37-wt and is similarly distributed to the triton X-100 insoluble protein fraction. When coexpressed with Cx43, antibodies against either connexin co-precipitate both connexins, consistent with heteromeric channel formation of both Cx37wt and Cx37-T154A with Cx43. Dual whole-cell voltage clamp studies of Cx37wt type expressing Rin cells reveal a coupling incidence of 50-60%, no coupling is observed in Cx37-T154A expressing cells. In addition, introduction of Cx37-T154A into a Cx43-expressing MDCK cell line reduced the coupling level in these cells (dye coupling studies are underway), consistent with a dominant negative function of a channel-dead form of Cx37. Contrary to our hypothesis, Cx37-T154A expressing cells were not growth suppressed, suggesting a functional pore domain is necessary for growth suppression by Cx37. Furthermore, whereas cell cycle duration was significantly increased (relative to Cx-deficient Rin cells) in Cx37wt expressing Rin cells and cells accumulated in G1, cell cycle duration was unchanged in cells expressing Cx37-T154A and accumulation in G1 did not occur. The present data thus suggest that a functional pore-forming domain is essential for the anti-proliferative effects of Cx37. Supported by: HL064232 and AHA 550158Z.

2386/B93

Characterization of Pannexin Intracellular Interactions.

S. Penuela[1], R. Bhalla[1], S. J. Celetti[2], Q. C. Shao[1], D. W. Laird[1,2]; [1]Anatomy and Cell Biology, University of Western Ontario, London, ON, Canada, [2]Physiology and Pharmacology, University of Western Ontario, London, ON, Canada

Panx1, Panx2, and Panx3 have been shown to form functional single membrane channels and even gap junction channels in some reference cell systems. In vivo, combinations of these pannexins are likely expressed within the same cell type raising the possibility that they may co-oligomerize to form unique channel properties. In vitro, confocal imaging revealed co-localization of Panx1 and Panx3 at the cell surface, while in vivo these pannexins have been demonstrated to acquire both overlapping and distinct localization profiles. To test their ability to interact in keratinocytes and 293T cells, different combinations of Panx1 and Panx3, along with their corresponding N-glycosylation-deficient mutants, were co-expressed. Immunofluorescent assays suggest that Panx1 interacts with the ER-retained Panx3-GFP mutant and partially rescues its trafficking defect while the glycosylation-deficient mutant of Panx1 interacts with Panx3-GFP and is more potently retained within intracellular compartments. Co-immunoprecipitation assays revealed the interaction of the ER-retained Panx3-GFP with Panx1 and its glycosylation-deficient mutant, further supporting the potential interaction between Panx1 and Panx3, specifically when enriched in the ER. In functional assays, although the glycosylation-deficient mutants exhibit impairment in trafficking, rhodamine dye uptake studies revealed that these mutants were still capable of forming functional single membrane channels in 293T cells. To aide in Panx1 and Panx3 localization and functional studies, additional antibodies to the cytoplasmic and extracellular loops (EL) of these pannexins were generated that recognized the previously reported species of Panx1 and Panx3 in Western blots. An anti-Panx3 EL antibody was further found to uniformly label fixed Panx3 expressing cells, and is thus a candidate for blocking single membrane Panx3 channel function in live cells. Collectively, our findings suggest that Panx1 and Panx3 can potentially interact to generate unique channel properties or function independently in distinct cellular compartments. Supported by the Canadian Institutes of Health Research.

2387/B94

Pannexin 1 and Pannexin 3 Have Distinct Epidermal Localization Profiles and Are Implicated in Keratinocyte Differentiation.

S. J. Celetti[1], K. N. Cowan[2,3], S. Penuela[3], Q. C. Shao[3], S. Langlois[3], B. J. Cowan[4], D. W. Laird[1,3]; [1]Physiology & Pharmacology, University of Western Ontario, London, ON, Canada, [2]Surgery, University of Western Ontario, London, ON, Canada, [3]Anatomy & Cell Biology, University of Western Ontario, London, ON, Canada, [4]Dermatology and Skin Science, University of British Columbia, Vancouver, BC, Canada

Pannexins (Panxs) are a small family of integral membrane proteins that have sequence homology to the innexin family of invertebrate gap junction proteins. Panxs form functional mechano-sensitive, single membrane channels but the importance of these channels in regulating cellular function is poorly understood. We have recently shown that Panx1 and Panx3 are expressed in mouse skin but their function in keratinocytes remains to be elucidated. Here we show that Panx1 and Panx3 are immunolocalized to the epidermis and multiple adenexal structures in intact human skin. Panx1 demonstrated a punctate, plasma membrane-like distribution throughout the layers of the epidermis, while Panx3 displays diffuse cytoplasmic labeling. This suggested that these proteins may play distinct roles within keratinocytes. Both Panxs were also prominent in keratinocyte-derived skin appendages, namely the hair follicle, where the subcellular localization of Panx1 became uniquely cytoplasmic. To establish a role for Panxs in keratinocyte differentiation, we used rat epidermal keratinocytes (REKs) that possess the ability to differentiate into organotypic epidermis. Compared to monolayer cultures of REKs, the 70 kDa species of Panx3 was

greatly increased in organotypic epidermis, while Panx1, already expressed at a low level, exhibited a further decrease. To characterize the role of epidermal Panxs, we engineered REKs to stably over-express Panx1 or Panx3. Panx1 localized uniformly to the plasma membrane while Panx3 displayed both intracellular and plasma membrane profiles. REKs over-expressing Panx1 or Panx3 were significantly less proliferative, and when grown into organotypic epidermis, preliminary data revealed that the overall epidermal thickness was reduced, suggesting that Panxs may play a role in regulating keratinocyte growth and differentiation. In summary, Panx1 and Panx3 are co-expressed in the epidermis and their strict regulation appears to be necessary for normal keratinocyte proliferation and epidermal differentiation. Supported by the CIHR to DWL, LRCP to KNC, and NSERC to SC.

2388/B95

Chemical Gating Mechanism of Connexin26-Containing Channels by Aminosulfonate.

L. Tao[1], F. Kieken[2], J. Liu[1], P. Sorgen[2], A. Harris[1], D. Locke[1]; [1]Pharmacology and Physiology, New Jersey Medical School, UMDNJ, Newark, NJ, [2]Biochemistry and Molecular Biology, University of Nebraska Medical Center, Omaha, NE

Protonated aminosulfonates, including taurine, directly and reversibly inhibit homomeric and heteromeric Cx26-containing hemichannels but not homomeric Cx32 hemichannels (JBC 274:3711 & 279:38544). It is unknown which connexin domains are involved, if taurine interacts with Cx26 and/or Cx32 in heteromeric channels or if junctional channels are taurine-sensitive. These issues were addressed with channels composed of Cx26 and/or Cx32 with/without a cleavable 3KDa carboxyl-terminal (CT) tag (T). Hemichannel activity was assessed in liposomes, and in cells by extracellular dye uptake. In contrast to untagged hemichannels, in both assays, Cx26T/Cx32 and Cx26T hemichannels were not taurine-sensitive, but Cx26/Cx32T hemichannels were. Tag cleavage (Tc, leaving 4aa at the carboxyl-terminus) restored the inhibition of Cx26Tc/Cx32 hemichannels, but taurine surprisingly narrowed rather than closed Cx26Tc hemichannels. Thus, a large Cx26 CT tag blocks taurine-sensitivity, unless hemichannels also contain Cx32, and a short 4aa CT extension affects the open state of Cx26Tc channels. Taurine effects on junctional channels were assessed by intercellular dye-coupling. Taurine substantially reduced dye transfer by Cx26 and Cx26/Cx32T channels, but not by Cx26T, Cx26T/Cx32 or Cx32T channels. Junctional channels therefore have the same taurine-sensitivity as their component hemichannels. An intracellular site for taurine action was shown with a membrane-impermeable blocker of taurine uptake. Thus, all data indicate taurine-induced pore closure utilizes the Cx26 CT. Binding of taurine to the Cx26 CT was assessed by natural abundance 13C-HMQC NMR. Overlapping resonances of CT peptide in the presence and absence of taurine indicate no direct taurine binding to CT. pH gating of Cx43 channels involves the cytoplasmic loop (CL) binding CT. Peptide 'elisa' showed that a pH dependent interaction also occurs between Cx26 CT and the carboxyl-terminal 20aa of the Cx26 CL. Acidification increases the binding affinity of CL and CT peptides, and only the protonated form of taurine negatively affects the interaction. Structural analysis of Cx26 CT and CL peptides in the presence and absence of taurine are ongoing. Supported by GM36044, DC7470.

2389/B96

Connexin Pore Block by ABG-Sugars.

D. Locke[1], F. Sprenger[2], S. Spreitz[2], A. Harris[1]; [1]Pharmacology and Physiology, New Jersey Medical School, UMDNJ, Newark, NJ, [2]AGLYCON, Grambach, Austria

Pore blockers are of great value for structure-function study of membrane channels. Prior work shows that novel anthranilamide moieties (ABG) derivatized to maltosaccharides of different lengths (Gn: n glucose units) are size-indexed pore blockers of reconstituted connexin channels: block occurs with precise size-match with a segment of the pore lumen, not if the lumen or blocker is too narrow or wide. Permeation studies using the same maltosaccharides derivatized with a small uncharged fluorescent group (PA-sugars) show the narrowest part of the pore (size-selective filter) decreases in order Cx32>Cx26≈Cx26/Cx32, the last form being heteromeric (JBC 273:2808 & ECR 298:643). The present studies of the efficacy of ABG-sugar block from each side of the pore reveal new information about channel structure. The different connexin pores have constricted segments with which ABG-sugars interact, other than the size-selective filter. From the cytoplasmic aspect of the pore, ABG-G3 blocks Cx26 but not Cx32 or Cx26/Cx32 channels, and ABG-G4 blocks with decreasing effectiveness Cx32>Cx26>Cx26/Cx32. From the extracellular side, ABG-G3 blocks Cx26/Cx32 better than Cx26, and does not block Cx32 channels, while ABG-G4 has no effect on any channels tested. If block were exclusively at the size-selective filter, the pattern of block from both sides of the pore should be identical and consistent with the pore sizing study. Instead, block by ABG-sugars indicates that pore width varies as the selectivity filter is approached from one side or the other. Specifically, there is narrowing of the pore lumen of homomeric Cx26 and Cx32 channels on the cytoplasmic side of the selectivity filter. Intriguingly, heteromeric Cx26/Cx32 channels show a unique and substantial narrowing extracellular to the selectivity filter. This is significant as most connexin channels in vivo are heteromeric, and heteromeric channels show profound selectivity amongst second messengers that the corresponding homomeric channels do not (JBC 273:2808, 218:16727 & ECR 298:643). Our data suggest that a consequence of 'heteromericity' is that segment/s of a pore-lining domain are asymmetrically displaced toward the center of the lumen. Supported by GM36044, NS56509.

2390/B97

Posttranslational Modifications of Connexin26 Identified by Maldi-TOF/TOF Mass Spectrometry.

S. Bian[1], H. Li[2], J. Liu[1], A. Harris[1], D. Locke[1]; [1]Pharmacology and Physiology, New Jersey Medical School, UMDNJ, Newark, NJ, [2]Center for Advanced Proteomics Research, New Jersey Medical School, UMDNJ, Newark, NJ

Gap junctions play important roles in auditory function. Mutations in the Cx26 gene are the predominant cause of inherited nonsyndromic deafness. Some Cx26 deafness mutations directly disrupt the intercellular molecular and/or ionic signaling pathway, while others may affect the location and character of posttranslational modifications (PTMs) governing channel assembly, function and biological regulation. Mass spectrometry was used to determine if Cx26 PTMs occur at sites of deafness-causing mutations. Cx26 was isolated from HeLa cells at >95% purity by immunoaffinity followed by metal chelate chromatography using carboxyl-terminal hexahistidine and hemagglutinin tags. In-gel and in-solution enzymatic digestions were carried out in parallel with trypsin, chymotrypsin and endoproteinase-GluC. Peptides were recovered with and fractionated from reverse-phased C8 beads by stepwise elution with increasing concentrations of organic solvent. Using an ABI4800 MALDI-TOF/TOF-MS, spectra were acquired from each elution step, thereby improving detection of low abundance peptides in

complex mixtures and maximizing sequence coverage. Acquisition, processing and interpretation parameters were further optimized to improve ionization and fragmentation of hydrophobic connexin peptides. MALDI-TOF-MS and MALDI-TOF-MS/MS sequence coverage values obtained were significantly above those reported for other mammalian membrane proteins. Total Cx26 sequence coverage by MS was 75.2%, with 31.1% sequence confirmed by MS/MS. Improved ionization and sequencing of Cx26 peptides (especially transmembrane pore-lining domains) were further achieved with a Waters nano-liquid chromatography-coupled electrospray ionization quadrupole-TOF-MS. Several different PTMs of Cx26 were identified, many of which were at sites of deafness-causing mutation. The PTMs included phosphorylation, acetylation, methylation, citrullination, hydroxylation, γ-carboxylation and palmitoylation. Knowledge of the location and character of Cx26 PTMs will be instrumental in guiding experiments to understand how cellular mechanisms of channel regulation can become altered and lead to losses in auditory function. Supported by GM36044, DC7470, NS56509 (ALH) & NS046593 (HL).

2391/B98

Molecular Characterisation of Pannexin-1 and Pannexin-3 in the Male Reproductive Tract of Adult Rat.

P. Turmel[1,2], l. hermo[1], C. E. Smith[3], S. Penuela[4], D. W. Laird[4], D. Cyr[1,2]; [1]Anatomy and Cell Biology, McGill University, Montreal, QC, Canada, [2]INRS-Institut Armand Frappier, Université du Québec, Laval, QC, Canada, [3]Départment de Stomatologie, Université de Montréal, Montreal, QC, Canada, [4]Department of Anatomy and Cell Biology, University of Western Ontario, London, ON, Canada

Recently, a novel gap junctional family of proteins, the pannexins (panxs), has been identified. Although panxs are known to be the vertebrate homologs of invertebrate gap junction proteins, innexins, their function in mammals is controversial and remains obscure. Three panx members have been identified to date and their localization and functions have already been examined in certain tissues, such as the brain and skin. However, very little is known relative to their involvement in the male reproductive tract. To test the possibility that panxs play a role in this organ, we have investigated the expression and distribution of panx-1 and -3 in the testis, efferent ducts, and epididymis of adult rat using immunohistochemistry and immunofluorescence. In testis, panx-3 labelling was found to be punctate in areas of Leydig cells. The localization of panx-1 in the testis remains unclear and warrant further investigations. In efferent ducts, panx-1 and panx-3 appear to be expressed mainly in epithelial ciliated cells. In the epididymis, both panxs were shown to be expressed by principal cells and were localized in the cytoplasm and at the plasma membrane. In the case of panx-1 only, prominent labelling was also observed between principal and basal cells. Conversely, clear cells do not seem to express any panx. It is noteworthy that the expression level of panx-1 varied along the different regions of the epididymis suggesting that its functions may be region specific. We have also examined tissue distribution of panx-1 isoforms by Western blots analyses. Numerous isoforms of various molecular weights (44-61kDa) were observed in the testis and all regions of the epididymis, corroborating with previous evidences of post-translational glycosylation. However, preliminary results suggest that some of the isoforms observed might also be due to alternative splicing. In conclusion, panxs are expressed throughout the male reproductive tract and their expression is both tissue and cell type specific. Moreover, the differential expression of panxs in the epididymis suggests that they may be tightly regulated within this tissue and may thus play a role in the process of epididymal sperm maturation. Supported by CIHR and NSERC.

2392/B99

Calphostin C Induced Cortical Astrocyte Death Is Mediated by Hemichannels Formed by Pannexin1.

N. Vega[1], P. Lampe[2], L. Reuss[3], J. C. Sáez[1]; [1]Depto. de Ciencias Fisiológicas, Pontificia Universidad Católica de Chile, Santiago, Chile, [2]Molecular Diagnostics Program, Fred Hutchinson Cancer Research Center, Seattle, WA, [3]Cell Physiology & Molecular Biophysics, Texas Tech University Health Sciences Center, Lubbock, TX

Calphostin C (CC), a PKC inhibitor activated by light, induces cell apoptosis in a PKC independent way, and its molecular mechanism remains largely unknown. Because most cells express connexin43 (Cx43) and pannexin1 (Px1), which form hemichannels (HCs) permeable to ions and small molecules and their over activation might accelerate cell death, the present work was undertaken to evaluate their possible involvement in CC induced astrocyte apoptosis. Cultures of neonatal rat cortical astrocytes were used. Relative levels of surface Cx43 and Px1 HCs were evaluated by biotinylation and immunoblotting. Functional HCs were evaluated in time lapse etidium (Etd) uptake measurements. With Fura-2 AM, the intracellular free Ca^{2+} concentration ($[Ca^{2+}_i]$) was measured. Cell death was evaluated by propidium iodide uptake and blebs formation. CC rapidly (minutes) increased the $[Ca^{2+}_i]$ followed by a progressive increase in Etd uptake cell surface levels of glycosilated Px1 HCs, reduced levels of surface Cx43 HCs and caused cell death, all within 30 min. The Etd uptake was partially sensitive to 200 μM La^{3+} and 35 μM 18β-glycyrrhetinic acid, suggesting the involvement of both, Cx43 and Px1 HCs. But, all changes were prevented with tunicamycin or brefeldin A, a N-glycosidase and trans Golgi transport blocker, respectively, or with ^{10}Px1, a mimetic peptide blocker of Px1 but not Cx43 HCs. Moreover, La^{3+}, a Cx43 but not Px1 HCs blocker, did not prevent the CC induced effects. The CC induced Etd uptake rise was not observed in cells loaded with BAPTA and in zero $[Ca^{2+}_o]$. We propose that CC activates a positive feedback mechanism, starting with a rise in $[Ca^{2+}_i]$ which increases the insertion of Px1-HCs, activation of both HC types, promotion of Ca^{2+} influx leading to intracellular Ca^{2+} overload and cell death.

2393/B100

Evidence of Pannexin1 Hemichannels in Murine T Lymphocytes.

K. F. Shoji[1,2], P. J. Sáez[1,2], H. A. Sanchez[1,2], J. Saez[1,2]; [1]Department of Physiology, Pontificia Universidad Catolica de Chile, Santiago, Chile, [2]Núcleo Milenio de Inmunología e Inmunoterapia, Santiago, Chile

In murine T lymphocytes extracellular ATP induces opening of a highly permeable channel followed by cell death. Until now, it is accepted that the purinergic receptor $P2X_7$ is the molecular identity of this channel. Moreover, a recent report in macrophages has demonstrated that $P2X_7$ channels are not permeable to ethidium (Etd). In fact, it demonstrates that the ATP induced Etd uptake is mediated by pannexin1 hemichannels (Px1 HCs). Thus, the present work was undertaken to study the possible involvement of Px1 HCs in T cell death induced by extracellular ATP. We used freshly isolated murine T lymphocytes from lymph nodes. The presence and cellular distribution of Px1 was evaluated by immunoblotting and immunofluorescence, respectively. The activity of HCs was evaluated in time lapse Etd uptake measurement and electrophysiologic studies using whole cell voltage patch clamp. Cell death was evaluated with rhodamine-dextran (10

kDa) labeling. Px1 was detected at the cell membrane in both helper ($CD4^+$) and citotoxic ($CD8^+$)T lymphocytes. It was also detected in lymph nodes, spleen, Peyer patches and thymus by Western blot and inmunofluorescence analysis. The ATP induced Etd uptake was partially inhibited by 5μM carbenoxolone (CBX), 1mM probenecid or the mimetic peptide ^{10}Panx1 peptide, all known to inhibit Px1 HCs. In resting T lymphocytes, Px1 like unitary current events with unitary conductance of ~500pS were identified. Moreover, the ATP induced cell death was partially prevented with probenecid and CBX. Thus, we propose that T cells express Px1 HCs and their activation mediate T cell death triggered by extracellular ATP.

2394/B101

The Pore Structure of Pannexin 1 Channels.

J. Wang, G. Dahl; Physiology and Biophysics, University of Miami, Miami, FL

Vertebrates express two families of gap junction proteins, the well characterized connexins and the pannexins. In contrast to connexins, pannexins do not appear to form gap junction channels but instead function as unpaired membrane channels. Pannexins have no sequence homology to connexins, but are distantly related to the invertebrate gap junction proteins, innexins. Despite the sequence diversity, pannexins and connexins form channels with similar properties and exhibit similar membrane topology. To test whether the similarities extend to the pore structure of the channels, pannexin 1 was subjected to analysis with the Substituted Cysteine Accessibility Method (SCAM). The thiol reagents MBB and MTSET reacted with several cysteines positioned in the external portion of the first transmembrane segment (TM1) and the first extracellular loop. In addition, MTSET reactivity was found in the internal portion of TM3. These data suggest that Portions of TM1, the first extracellular loop and TM3 line the pore of pannexin 1 channels. Thus, the pore structures of pannexin 1 is similar to that of connexin channels.

2395/B102

A Permeant Regulating Its Permeation Pore: Inhibition of Pannexin 1 Channels by ATP.

G. Dahl, F. Qiu; Physiology and Biophysics, University of Miami, Miami, FL

Pannexin 1 forms a large membrane channel that based on its biophysical properties and its expression pattern, is a prime candidate to represent an ATP release channel. Pannexin 1 channel activity is potentially deleterious for cells as indicated by its involvement in the P2X7 death complex. Here we describe a negative feedback loop controlling pannexin 1 channel activity. ATP, permeant to pannexin 1 channels, was found to inhibit its permeation pathway when applied extracellularly. ATP analogues, including BzATP (Benzoylbenzoyl-ATP), suramine, and BBG (Brilliant Blue G) were even more effective inhibitors of pannexin 1 currents than ATP. These compounds also attenuated the uptake of dyes by erythrocytes, which express pannexin 1. The rank order of the compounds in attenuation of pannexin 1 currents was similar to their binding affinities to the P2X7 receptor, except that receptor agonists and antagonists both were inhibitory to the channel. Mutational analysis identified R75 in pannexin 1 to be critical for ATP inhibition of pannexin 1 currents.

2396/B103

Pannexin 1 Activates the Inflammasome in Neurons and Astrocytes.

W. Silverman, S. Locovei, J. de Rivero Vaccari, G. Dahl, R. Keane; Physiology and Biophysics, University of Miami, Miami, FL

Pannexin 1 forms the large pore of the P2X7 purinergic receptor. P2X7 is involved in inflammation, and activation of P2X7 by extracellular ATP results in processing of interleukin-1 by the inflammasome. Pannexin 1 is opened by positive membrane potentials, yet cells that express pannexin 1 release ATP in response to extracellular K+ at potentials that are not sufficiently polarized for activation of the voltage gate. To investigate this apparent discrepancy, we have looked at the role of extracelular K+ in the opening of pannexin 1 channels and the activating the inflammasome. Our results indicate that extracellular K+ results in activation of pannexin1 channels independent of voltage. Extracellular K+ also activated the inflammasome in neurons and astrocytes, and inhibition of pannexin 1 lead to attenuation of caspase-1 activation . In addition neutralization of the inflammasome blocked ATP-induced cell death. Thus, it appears that high extracellular K+ in excitable cells results in inflammasome activation, in contrast to macrophage and monocytes, where low intracellular K+ is viewed as the trigger for inflammation.

2397/B104

Electron Microscopic Characterization of isolated Pannexin Channels.

C. Ambrosi, D. Boassa, J. N. Pranskevich, A. Smock, G. Sosinsky; National Center for Microscopy and Imaging Research (NCMIR), University of California, San Diego, La Jolla, CA

Connexins (Cx) are widely studied channels in intercellular communication (gap junction) and allow exchange of molecules less than 1000 Da between neighboring cells. Pannexins (Panx1, Panx2 and Panx3) have been hypothesized to have a similar protein folding topology, but show no sequence homology with connexins. Pannexin channels are set apart from connexins by several of their physiological attributes: large single channel conductances, ability to readily make hemichannels rather than intercellular channels, glycosylated forms and lack of sensitivity to extracellular Ca2+. When expressed in mammalian cells, pannexins do not form canonical gap junctions and may be found as oligomers either in the plasma membrane or in the cytoplasm. We applied methods for purification and structural analysis, developed for connexin-based gap junctions, to rat Panx1 and rat Panx2. By electron microscopy (EM) we have been able to identify cellular membranes that clearly show pannexin channels when isolated from MDCK cells stably expressing rPanx1 or rPanx2. Immunolabeling by secondary antibodies colloidal gold-conjugated for EM and Western blots for protein analysis, confirmed that rPanx1 or rPanx2 form doughnut-like structures (pannexons). Isolated membranes from parental HeLa cells that do not endogenously express rPanx1 or rPanx2 and are communication deficient did not contain any channel structures, failed to label with immunogold and lacked pannexin bands on Western

blots. Purified pannexons from both rPanx1-V5-His6-baculovirus and rPanx2-V5-His6-baculovirus infected Sf9 cells confirmed the doughnut-like structures observed in mammalian membranes and are comparable in size to baculovirus-expressed connexin46 and connexin50 hemichannels. Furthermore, we observe an increase in oligomer diameter that is correlated with an increase in subunit molecular weight among the five connexin hemichannels we have imaged. With larger oligomers such as those formed by Panx2 and Cx50, we see a more heterogeneous appearance of channels perhaps due to differential staining of different oligomer orientations. Pannexons and connexons expressed and purified in Sf9 cells represent a useful tool for size and structural analysis already in progress.

2398/B105

The Extracellular ATP Required for Potentiation of the Adult Skeletal Muscle Contraction Is Released through Pannexin 1 Hemichannels.

M. A. Riquelme, L. A. Cea, J. C. Sáez; Faculty of Biological Science, Department of Physiology, Pontificia Universidad Católica de Chile, Santiago de Chile, Chile

During repetitive skeletal muscle contractions a rise in extracellular ATP levels activates purinergic ionotropic receptors (P2X), increasing the Ca^{2+} influx and enhancing the contraction force, response called potentiation. Pannexins (Pxs) belong to a family of proteins composed of 3 members (Px1-3). Px1 forms homomeric hemichannels (Px1-HCs) permeable to small molecules such as ATP and ethidium (Etd) and only Px1 is expressed in skeletal muscle. The ATP-release pathway during potentiation remains unknown and Px1-HCs are good candidates. The presence of Px1 was studied in *Tibialis Anterior* (*TA*) muscles (immunoblot) and in *Soleus* and isolated fibers from *Flexor Digitorium Brevis* (*FDB*) muscle (immunoflorescence). The functional state of Px1-HCs was evaluated in *FDB* (time lapse of Etd uptake). Levels of extracellular ATP of *Soleus* and *Extensor Digitorium Longus* (*EDL*) muscles were measured with a luciferin/luciferase assay. In total protein extracts, the Px1 monomer (~43 kD) and aggregates were detected and competed with the immunogenic peptide. Px1 immunoreactivity was found at T-tubules intercalated with dihydropyridine or ryanodine receptors. Connexins (Cxs) 32, 43 and 45 were not detected in muscle fibers. Under repetitive electrical stimulation occurs Etd uptake that was inhibited by β-glycyrrhetinic acid (βGA) or oleamide, blockers of both Px1- and Cx-HCs, but was insensitive to La^{3+}, a Cx-HC blocker. Electrical stimulation enhanced the ATP release from *Soleus* and *EDL* muscles, a response prevented by βGA and oleamide. HC blockers inhibited the electrical stimulation (<0.033 Hz) elicited potentiation and contraction remained at baseline levels. We propose that Px1-HCs open during repetitive contraction and allow the release of ATP required for potentiation of adult rat skeletal muscle contraction.

Structure and Function of Membrane Proteins (2399 – 2420)

2399/B107

Hyposialylation in Various Glomerulopathies and Possible Rescue by N-Acetylmannosamine.

J. Poling[1], I. Manoli[1], E. Klootwijk[2], D. Kurland[1], C. Ciccone[1], K. Patzel[1], D. Darvish[3], P. Zerfas[4], M. Starost[4], H. Dorward[1], W. A. Gahl[1], M. Huizing[1]; [1]Medical Genetics Branch, NHGRI/NIH, Bethesda, MD, [2]Dept of Medicine, University College, London, United Kingdom, [3]HIBM Research Group, Encino, CA, [4]Division of Veterinary Resources, NIH, Bethesda, MD

Mutations in UDP-GlcNAc 2-epimerase/ManNAc kinase (GNE/MNK), the bifunctional, rate-limiting enzyme in sialic acid (SA) biosynthesis, result in hereditary inclusion body myopathy (HIBM), an adult-onset, progressive myopathy. We created a knock-in mouse mimicking the human GNE/MNK Persian-Jewish M712T founder mutation. Homozygous mutant mice (GneM712T/M712T), do not survive (with one exception) beyond postnatal day 3 (P3), with the only noted pathology in the kidneys. GneM712T/M712T mice have grossly hemorrhagic kidneys, with podocyte foot process effacement, glomerular basement membrane (GBM) splitting, and hyposialylation of podocalyxin (PDXN), the primary podocyte sialoprotein; these changes result in hematuria, proteinuria, and elevated serum creatinine and BUN. Expression of other glomerular compartment markers (laminin β1, VSMA, ezrin, and desmin) was unchanged. Previous studies indicated that loss of SA residues on PDXN resulted in disruption of its interaction with the actin cytoskeleton (via ezrin and NHERF2) in podocytes; this disrupted interaction or general loss of charged residues could explain this phenotype in GneM712T/M712T pups. Oral intake of the SA precursor ManNAc (the SA precursor immediately downstream of the epimerase activity) increased survival beyond P3 in 43% of GneM712T/M712T pups, with improvements in glomerular architecture and podocalyxin sialylation. Hyposialylation has been suggested in several studies examining unexplained human glomerulopathies: renal biopsies from minimal change disease patients showed decreased SA staining along podocyte membranes; membranous glomerulonephritis biopsies showed decreased SA along podocytes and the lamina densa externa of the GBM; and diabetic glomerulosclerosis biopsies showed decreased SA in the GBM (although this finding remains controversial). Based on the increased survival and partial restoration of glomerular architecture in our ManNAc-treated mice, ManNAc might be considered as a potential treatment in humans afflicted by renal disorders characterized by hyposialylation of the glomerular filtration apparatus.

2400/B108

Survey and Characterization of Single Nucleotide Polymorphisms of SLC22A11 (hOAT4) in Korean Women Osteoporosis Patients.

S. Cha[1], W. Lee[2], H. Hwang[3]; [1]Pharmacology and Toxicology, Inha University, Incheon, South Korea, [2]The DNA Laboratory, Hankook Life Science, Seoul and Wonju, South Korea, [3]Physiology and Biophysics, Inha University, Incheon, South Korea

Single nucleotide polymorphisms (SNPs) are the most common form of human genetic variation. The non-synonymous SNPs (nsSNPs) cause changes in certain amino acid sequence that are likely to be failed to show the functional diversity of encoded proteins. The organic anion transporters (OATs) play an important role for elimination or reabsorption of endogenous or exogenous organic anionic compounds. Among OATs, hOAT4 mediated the high affinity transport of estrone sulfate and dehydroepiandrosterone sulfate. The rapid bone loss that occurs in post-menopausal women is mainly due to the net decrease of estrogen. From 50 healthy subjects and 50 subjects with osteoporosis we screened for genetic polymorphism in coding region of SLC22A11 (hOAT4) by denaturing gradient gel electrophoresis (DGGE). Using GC-clamp PCR and DGGE assay, we were able to find three SNPs of hOAT4 in Korean women osteoporosis and normal subjects. Two of

these SNPs were found in osteoporosis group (C483A and G832A) and one of them in normal group (C847T). Among these SNPs, G832A was nsSNP that change 278th amino acid from glutamic acid to lysine (E278K). The uptake of [3H] estrone sulfate via hOAT4 mutant E278K was reduced when compared with wild-type hOAT4. The Km value for wild type and E278K was 0.7 micro M and 1.2 micro M, and the Vmax for wild-type and E278K was 1.8 and 0.47 pmol/oocyte/h, respectively. The present study demonstrates that hOAT4 variants are causing to inter-individual variations that is leading to the differences in anionic drug disposition and perhaps used as a marker to certain disease including osteoporosis.

2401/B109

Cloning and Characterization of Cationic Amino Acid Transporter 5.

T. Kimura[1], M. Kanai-Azuma[2], Y. Li[3], N. Anzai[1], H. Kawakami[2], H. Endou[4], H. Sakurai[1], Y. Kanai[3]; [1]Department of Pharmacology and Toxicology, Kyorin University School of Medicine, Tokyo, Japan, [2]Department of Anatomy, Kyorin University School of Medicine, Tokyo, Japan, [3]Department of Pharmacology, Graduate School of Medicine, Osaka University, Osaka, Japan, [4]J-Pharma Co. Ltd., Tokyo, Japan

We identified a novel Na^+-independent cationic amino acid transporter (CAT) with distinct characteristics in substrate selectivity and transport properties in mice. The newly identified protein, designated CAT5, belongs to SLC7 family and shows remarkable sequence similarity to those of mouse cationic amino acid transporters CAT1 (55 % identity), CAT2a (52 %), CAT3 (65 %) and CAT4 (42 %). When CAT5 was expressed in Xenopus oocytes, it transported basic amino acids such as arginine, lysine and ornithine with high affinity, consistent with the properties of basic amino acid transport system y^+. Distinct from already known system y^+ isoforms, CAT5 mediated Na^+-independent transport of large neutral amino acids with low affinity. Multiple tissue Northern blot revealed that CAT5 mRNA expression was only in the placenta and immunohistochemistry showed that CAT5 was exclusively expressed in syncytiotrophoblast of the placenta and yolk sac. In the yolk sac, CAT5 was co-localized with a nitric oxide synthase eNOS, caveolin-1 and caveolin-2. To confirm whether CAT5 exists in the caveolae fraction, placenta proteins were separated by sucrose gradient centrifugation and probed for CAT5 and caveolae markers. CAT5 went to the fraction same as caveolae markers such as Caveolin-1, Caveolin-2, flotillin-1 and eNOS but expression pattern of CAT5 was different from the non-caveolae marker, clathrin. We examined the effect of caveolin on cell surface expression of CAT5 in NIH3T3 cells. When NIH3T3 cells were transfected with CAT5 alone, CAT5 was expressed primarily at the cell surface. Over-expression of caveolin-1 and caveolin-2 had very little effect on subcellular localization of CAT5. In the presence of caveolin-1 dominant negative mutants, however, CAT5 was redistributed away from the cell surface and was co-localized with these mutants. This result indicates that cell surface expression of CAT5 is regulated by caveolin. Taken together, CAT5 is likely to be a major cationic amino acid transporter in the placenta and yolk sac. Interestingly, CAT5 may be involved in the nitric oxide (NO) synthesis in the yolk sac and its subcellular localization was regulated by caveolin.

2402/B110

Molecular Identification of a Kidney Specific Murine Organic Cation Transporter Like-1 (Moctl1).

S. CHA[2], C. Suh[1]; [1]Physiology and Biophysics, Inha University College of Medicine, Incheon, South Korea, [2]Pharmacology and Toxicolgy, Inha University College of Medcine, Incheon, South Korea

Recently discovered organic ion transporters are expressed in various tissues, which are all epithelial tissues that transport endogenous and exogenous compounds and their metabolites. There are organic anion transporter (OAT), organic cation transporter (OCT), organic anion transporter like protein (OATLP) and organic cation tansporter like (OCTL). When considering the variety of charged organic ionic compounds, the existence of other isoforms of organic ion transporters can be assumed. In this connection, we have searched for a new isoform in the expressed sequence tag (EST) database using hOAT4 amino acid sequence as a "query." We found a candidate clone (BC021449) from the mouse kidney cDNA library. This clone was identified ortholog of ORCTL3 or OCTL-1. The mOCTL1 cDNA consisted of 2016 base pairs that encoded a 551 amino acid residue protein with 12 putative transmembrane domains. The deduced amino acid sequence of mOCTL1 showed 35 to 40% identity to those of the other members of the OATs and OCTs. According to the tissue distribution with performing Northern blot analysis, about a 2.4 kb transcript of mOCTL1 was observed in the kidney. About a 90-kDa band was detected when Western blot analysis in the mouse kidney was done with using synthesized oligopeptide-induced mOCTL1 antibody. The immunohistochemical result showed mOCTL1 was stained at the glomerulus (the parietal epithelial cells and podocytes), pars recta of proximal tubule, distal convoluted tubules, connecting tubules and collecting tubules. From these results, mOCTL1 may be a candidate for an organic ion transporter isoform that was identified in the kidney.

2403/B111

Characterization of the Sperm-Specific NHE (NHE10) from Human and Rat.

J. Sanjeevi, P. James; Zoology, Miami University, Oxford, OH

NHE1, NHE5 and NHE10 are the three Na/H exchanger isoforms found in mouse sperm. NHE1-null mice fail to show any infertility phenotype, whereas NHE10-null mice are infertile due to severe sperm motility defects. Addition of NH4Cl to NHE10-null sperm partially restores motility by alkalinizing the sperm cytoplasm, suggesting that NHE10 plays a major role in maintaining sperm motility by regulating intracellular pH. However, no Na/H exchanger activity has been demonstrated for the wild type NHE10. In any case, the sperm-specific expression of this NHE and its evident importance in fertility makes it an attractive contraceptive target and since motility can be restored to NHE10-null sperm, it may serve as a target to treat infertility. The existence of the functional sperm- specific NHE10 in human and rat is unclear. Here, we provide evidence for the expression of NHE10 in human and rat. We have sequenced a hNHE10 cDNA, obtained as an EST from the I.M.A.G.E consortium. The human NHE10 peptide sequence shares 60% homology with the mouse peptide and like the mouse, it has 14 predicted transmembrane segments, a putative voltage sensor, and a cyclic nucleotide binding motif. We have also sequenced the rat NHE10 cDNA, obtained using RT-PCR from rat testes mRNA. Our rat NHE10 cDNA is longer than the rat NHE10 sequence predicted from genomic sequencing and its deduced amino acid sequence shares 90% homology with the mouse peptide. Like the mouse and human proteins, the rat NHE10 also has 14 predicted transmembrane segments, a putative voltage sensor, and a cAMP binding motif, whereas the rat NHE10 sequence predicted from the rat genome project would have just 8 transmembrane segments, and would not

have a cAMP binding motif. Here we demonstrate the testes-specific expression of these NHE10 mRNAs. In addition, we also demonstrate that NHE-deficient cells transfected with the hNHE10 cDNA are resistant to H+- killing, suggesting that at least the human NHE10 is a functional Na/H exchanger. Our future focus will be to characterize the activity of these NHEs and to compare the transport characteristics with other known NHEs

2404/B112

Localization of the "Sperm-Specific" Na,K-ATPase Isoform to Other Ciliated Cells in Human and Mouse Tissues.

D. L. Kumar, P. James; Zoology, Miami University, Oxford, OH

The Na,K-ATPase is an intergral membrane protein present in the plasma membrane of all higher eukaryotes. This P- type ATPase is minimally composed of an alpha subunit and a beta subunit. Isoforms for both alpha and beta subunits have been identified. Four alpha isoforms (alpha1, alpha2, alpha3 and alpha4) and three beta isoforms (beta1, beta2 and beta3) have been described in mammals. Being the catalytic subunit of the enzyme our focus is on the alpha subunit and in particular the alpha4 isoform. This isoform has been shown to be expressed only in the sperm flagellum of rats, mice and humans. However, since several proteins expressed in the sperm are also expressed in other ciliated cells as well, we hypothesized that alpha4 may be a ciliated/flagellated cell specific protein. To test this hypothesis we probed a human multi-tissue array using immunohistochemistry for the expression of the alpha4 isoform. Our results demonstrate the expression of this protein in ciliated cells of the fallopian tube and uterine glands. In addition, we have identified the expression of the alpha4 isoform in the ciliated cells of the mouse trachea and oviduct. Taken together these data suggest that the Na,K-ATPase alpha4 isoform may not be just a sperm specific protein but might be a ciliated/flagellated specific protein. Previous studies have shown that inhibition of the alpha4 isoform is sufficient to inhibit sperm motility in rat, mice and humans. Hence specific inhibitors of alpha4 could be developed as male contraceptive compounds. Identifying all possible sites of expression of alpha4 is therefore critical as inhibition of this protein elsewhere could have a detrimental effects not related to inhibition of sperm motility. Future studies will also involve looking at the role of this isoform in the function of cilia in tissues other than the testis.

2405/B113

Role of N-Glycosylation and N-Glycan Trimming of GABA Transporter 1.

H. Fan[1], Q. Cai[1,2], J. Hu[1], P. Salonikidis[1,3], J. Fei[1,2], W. Reutter[1], W. Schwarz[1,3]; [1]Charite-Universitätsmedizin, Berlin, Institut für Biochemie und Molekularbiologie, Berlin, Germany, [2]Institute of Biochemistry and Cell Biology, SIBS, CAS, Shanghai, China, [3]Max-Planck Institut für Biophysik, MPG, Frankfurt/M, Germany

GABA (γ−aminobutyric acid) is the major inhibitory neurotransmitter in the central nervous system (CNS). GABA appears to be involved in the control of anxiety, pain, anticonvulsive activity, cardiovascular regulation and depression. GABA re-uptake by GABA transporters from the synaptic cleft is one important mechanism in the regulation of GABA activity. The GABA transporter GAT1 belongs to the family of Na+- and Cl--coupled transport proteins, which possess 12 putative transmembrane domains and three N-glycosylation sites in the extracellular loop between transmembrane domain 3 and 4. To study the significance of N-glycosylation, green fluorescence protein (GFP)-tagged wild type GAT1 (NNN) and N-glycosylation defective mutants (DDQ, DGN, DDN, and DDG) were expressed in CHO cells. Compared with the wild type, all N-glycosylation mutants showed strongly reduced protein stability and trafficking to the plasma membrane, which however were not affected by 1-deoxymannojirimycin (dMM). This indicates that N-glycosylation, but not terminal trimming of the N-glycans is involved in the attainment of a correctly folded and stable conformation of GAT1. All N-glycosylation mutants were expressed on the plasma membrane, but they displayed markedly reduced GABA-uptake activity. Also, inhibition of oligosaccharide processing by dMM led to reduction of this activity. Electrical measurements revealed that terminal trimming of N-glycans plays a regulatory role in the GABA-translocation by affecting the affinity and the reaction steps associated with the sodium ion binding. The role of the terminal sialic acid of N-linked oligosaccharides of GAT1 in the GABA-reuptake is further investigated. CHO/GAT1 cells were treated with sialidase and GAT1 was expressed in CHOlec3 (a cell line with defection in sialic acid biosynthesis). In both cases deficiency of terminal sialic acid results in reduction of GABA-uptake activity significantly.

2406/B114

Disruption of Cajal Bands in Schwann Cells Does Not Affect Internodal Length.

D. Sherman, M. Grove, P. Brophy; Centre for Neuroscience Research, Edinburgh University, Edinburgh, United Kingdom

The Periaxin-DRP2-Dystroglycan (PDD) complex is responsible for the formation of appositions between the abaxonal surface of PNS myelin sheaths and the Schwann cell plasma membrane. These appositions delineate the cytoplasm-filled channels called Cajal bands. Mice lacking a functional Prx gene do not have appositions. They ensheath and myelinate axons in an apparently normal manner but the sheath subsequently destabilizes and the mice develop a severe demyelinating neuropathy. Surprisingly, the absence of Periaxin appeared to inhibit elongation during Schwann cell growth suggesting that intact appositions and therefore Cajal bands might regulate internodal lengths in the PNS. We have now tested this hypothesis by disrupting the PDD complex and deranging Cajal bands by inactivating the DRP2 gene. In contrast to the Periaxin-null mouse, DRP2-null Schwann cells have normal internodal lengths. This strongly suggests that the Prx gene has functions in addition to its role in assembling appositions and Cajal bands.

2407/B115

Human Glycolipid Transfer Protein (GLTP) Modulates Cell Shape.

Y. Gao, X. Zou, H. M. Pike, R. Brown; Hormel Inst-Univ Minnesota, Austin, MN

Glycolipid transfer proteins (GLTPs) are small (~24kDa), soluble, ubiquitously expressed proteins characterized by their ability to accelerate the intermembrane transfer of glycolipids in vitro. Over the past few years, substantial progress has been made towards understanding the conformational architecture of human GLTP and mapping of the glycolipid liganding site. The GLTP-fold is unique among lipid binding/transfer proteins and now serves as the prototype that defines the new GLTP superfamily. Nonetheless, the biological role of GLTP remains enigmatic. Here, we show that overexpression of GLTP in HeLa cells can markedly affect cell shape. More than half of transfected

cells overexpressing wtGLTP showed a rounded phenotype. However, HeLa cell shape was not altered by overexpression of point mutant W96A GLTP, a liganding-site alteration that almost completely blocks glycolipid transfer between membranes in vitro. Overexpression of GLTPD1, a newly discovered human GLTP-like orthologue that is unable to transfer glycolipids, also failed to elicit the rounded HeLa cell phenotype. Scratch/wound healing assays revealed that rounded HeLa cells overexpressing wtGLTP exhibited markedly decreased migration rates compared to cells with normal shape. Further studies into the mechanism by which GLTP modulates changes in cell shape are presently underway. [Support: NIH GM45928, NIH CA121493, & The Hormel Fnd.]

2408/B116

Defects in Cholesterol Transport Mediated by Mutations in NPC1.

K. L. Huegel, A. Rohly, P. Vaughan, K. Vaughan; Department of Biological Sciences, University of Notre Dame, Notre Dame, IN

Niemann Pick Type C (NPC) disease is a recessive neurodegenerative condition arising from defective transport and accumulation of cholesterol and other lipids in late endosomes and lysosomes. Of the two genes implicated in this disease (NPC1 and NPC2), NPC1 is a large multi-pass transmembrane glycoprotein of unknown function. Live cell imaging of GFP-tagged constructs reveals that wild-type and mutant NPC1 proteins incorporate into the same membranes that are positive for markers of late endosomes and lysosomes but negative for markers of the endoplasmic reticulum (ER) or other compartments. Live cell analysis indicates that the primary problem with mutant NPC1 proteins is the inability to generate tubulo-vesicular (NPC1-TV) membranes that emanate from late endosomes and lysosomes. These NPC1-TV membranes are positive for cholesterol and provide a novel mechanism of cholesterol transport between compartments in the endocytic pathway. Formation of NPC1-TV membranes required a dynamic microtubule (MT) network, MT tip-tracking proteins including dynactin, functional rab 8 and functional EHD1. Defects in any one of these components led to insufficient transport of cholesterol and accumulation of cholesterol in late endosomes and lysosomes. Wild-type NPC1 was retained in the ER after tunicamycin treatment, consistent with an important role for glycosylation in transport to late endosomes and lysosomes. Tunicamycin washout allowed normal trafficking of NPC1 from the ER to late endosomes and lysosomes and re-established NPC1-TV formation. Interestingly, the ability to reform NPC1-TV membranes correlated with localization of cholesterol to the ER where cholesterol levels in the cell are monitored. This suggests that NPC1-TV membranes play an important role in cholesterol efflux from late endosomes and lysosomes. These results suggest that NPC1 is responsible for the formation and activity of TV membranes that mediate cholesterol movement between the endocytic pathway and ER compartment. Because mutations in NPC1 are deficient in TV formation, this could explain the basis of defective cholesterol transport.

2409/B117

The Role of PKA in ABCA1-Mediated Cholesterol Efflux.

L. Ma, Y. Feng, M. Denis, J. Karwatsky, X. Zha; Chronic Disease, Ottawa Health Research Institute, Ottawa, ON, Canada

ATP-binding cassette transporter A1 (ABCA1) is known to mediate cholesterol and phospholipid efflux to lipid-poor apoA-I, a process that generates high density lipoprotein (HDL) particles. Recently, we have shown that expression of ABCA1 also leads to a basal level of cholesterol efflux in addition to HDL formation. This basal cholesterol efflux is independent of apoA-I. There are therefore two separate ABCA1-mediated pathways for cells to export cholesterol. The molecular mechanism by which ABCA1 mediates cholesterol efflux is still largely unknown. Previous studies have shown that PKA signaling is required for apoA-I dependent pathway of cholesterol efflux. We show here that the basal cholesterol efflux that is independent of apoA-I also relies on PKA activity. Both of these cholesterol efflux pathways are inhibited in the presence of a membrane permeable PKA specific inhibitor (PKI). However, we found that these two pathways require distinct compartmentalized PKA activities. PKA is thought to exert its localized function through anchoring proteins, A-kinase anchoring proteins (AKAPs) and manipulating PKA anchoring modulates many cellular functions. We used a short cell permeable peptide, st-Ht31, that disrupts plasma membrane PKA anchoring to AKAP79 and found that cholesterol efflux to apoA-I was completely abolished. However, we also observed that apoA-I independent basal cholesterol efflux was increased several folds with st-Ht31. This suggests that cytosolic PKA activity favors apoA-I-independent cholesterol efflux. We measured compartmentalized PKA activity using plasma membrane anchored and cytosolic FRET probes, pm-AKAR3 and c-AKAR3, respectively. We found that cytosolic PKA activity was greatly enhanced by st-Ht31. Our observations therefore demonstrate that the plasma membrane anchored PKA signaling is crucial for apoA-I dependent cholesterol efflux (HDL formation), while apoA-I independent cholesterol efflux preferentially requires cytosolic PKA signaling.

2410/B118

Oxidized LDL Binding Changes the Domain Organization of the Plasma Membrane.

L. L. Scheffer[1,2], C. L. Wheatley[1,2], E. L. Holicky[1,2], D. L. Marks[1,2], R. E. Pagano[1,2]; [1]Medicine, Mayo Clinic College of Medicine, Rochester, MN, [2]Biochemistry and Molecular Biology, Mayo Clinic College of Medicine, Rochester, MN

Oxidized low density lipoprotein (OxLDL) is believed to play an important role in atherogenesis by stimulating the proliferation and migration of smooth muscle cells (SMCs) to the atherosclerotic lesion. In human aortic SMC (HASMCs), some biological effects of OxLDL treatment occur within secs to minutes (e.g., generation of reactive oxygen species; stimulation of lactosylceramide synthesis). Accordingly, we hypothesized that OxLDL exerts these effects by modulating the organization of plasma membrane (PM) lipid microdomains, which in turn regulate processes as diverse as endocytosis and signaling. To test this hypothesis, we have used Bodipy-labeled sphingolipid (SL) analogs to monitor SL-enriched domains at the PM of living HASMCs. This was accomplished by simultaneously observing the monomer (green)/excimer (red) fluorescence emission of the SL analogs at the PM. Brief treatment with OxLDL strongly inhibited PM domain formation while normal LDL had no effect. Parallel, scanning electron microscopy studies demonstrated a dramatic reduction in the number of microvilli on the surface of OxLDL-treated cells relative to untreated cells or cells treated with normal LDL, suggesting a relationship between PM domains detected by fluorescent GSL analogs and PM protrusions. Additional experiments showed that: (i) pre-treatment of cells with α-tocopherol prevented the loss of excimer fluorescence following OxLDL treatment; (ii) incubation of cells with 7-ketocholesterol (a major oxysterol present in OxLDL) gave similar results to those found using OxLDL; and (iii) brief treatment of cells with OxLDL resulted in the formation of PM domains enriched in endogenous ceramide. These results support a model in which oxysterols are produced

and/or transferred to the PM subsequent to OxLDL binding, resulting in a change in the organization of PM lipids. Suppported by an AHA fellowship to LS and NIH Grant GM22942 to REP.

2411/B119

Caveolin-1 Is Required for the Normal Development of Cholesterol-Rich Lens Fiber Cell Membranes.

S. K. Biswas[1], M. H. Elliott[2], W. Lo[1]; [1]Anatomy & Neurobiology, Morehouse School of Medicine, Atlanta, GA, [2]Ophthalmology, Dean A. McGee Eye Institute, University of Oklahoma Health Sciences Center, Oklahoma City, OK

Objective: Caveolin-1 (cav-1) is a cholesterol-binding protein and plays an important role in regulation of membrane cholesterol transport. Here we report several significant changes in fiber cell membranes due to deficiency of caveolin-1 in the cav-1 null mice. Methods: Scanning, transmission and freeze-fracture TEM were used to examine morphological changes in fiber cell membranes of the cav-1 null mice as compared with the wild type at several ages. Filipin cytochemistry in conjunction with freeze-fracture TEM was applied to investigate the distribution of membrane cholesterol. Results: A gross examination revealed that both the eyeballs and lenses of cav-1 null mice were approximately 25% larger than those of the wild type. By scanning and transmission EM, the enlarged lenses in the cav-1 null mice were found associated with a significant increase in the volume of individual cortical fiber cells. The abnormal increase of the ridge (tongue-and-groove) surface pattern in the outer cortical fibers was also regularly observed in the young cav-1 null mice, but not in the wild type (e.g. 1 month old). In addition, freeze-fracture TEM showed that membrane particles (proteins) of cortical fiber cells were unevenly distributed, resulting in the formation of many large protein-free membrane areas (~4.15μm2) in the cav-1 null lens. Finally, filipin cytochemistry and freeze-fracture TEM demonstrated that these protein-free membrane areas contained enormous amounts of cholesterol (as represented by the filipin-cholesterol-complexes). It was estimated that the density of cholesterol was ~2.9 times higher in the protein-free areas than the adjacent protein-rich areas in a given cav-1 null lens. Conclusions: This study shows that the deficiency of cav-1 causes significant alterations in the lens size, membrane surface configuration and irregular distribution of membrane cholesterol in the cav-1 null mice. These results suggest that cav-1 is required for the maintenance of normal development and architecture of lens fiber cell membranes which contain a large amount of cholesterol.

2412/B120

Myristoylation of c-Src Regulates Src Kinase Activity, Stability and Ubiquitin Mediated Degradation.

P. P. Patwardhan, M. D. Resh; Cell Biology Program, Memorial Sloan Kettering Cancer Institute, New York, NY

Src family kinases (SFK) are a family of non-receptor tyrosine kinases that play a variety of roles in different cellular functions like cell adhesion, migration, proliferation, and stimulation of growth factor and cytokine pathways. The domain arrangement of SFK's consists of an N-terminal myristoylated SH4 region, followed by the unique region, the SH3, SH2 and catalytic domains and a short C-terminal tail. Myristoylation occurs co-translationally at the N-terminal glycine residue (Gly2) of all Src family members and is catalyzed by the enzyme N-myristoyl transferase (NMT). In this study, we observed that the expression level of a non-myristoylated mutant of c-Src (G2A) was 3 to 4 fold higher than that of wt c-Src in COS-1 cells. The non-myristoylated mutant accumulated in the cytosolic fraction. Treatment of transiently transfected COS-1 cells with 2-hydroxymyristate, an NMT inhibitor, also resulted in redistribution and accumulation of wt c-Src, thus mimicking the effects of G2A mutation. Expression levels of a constitutively activated form, c-SrcY527F, were lower than those of wt c-Src, but were increased when cells were treated with 2-hydroxymyristate. Despite higher expression, non-myristoylated c-Src exhibited decreased autokinase activity when assayed *in vitro* as well as when expressed in COS-1 cells. Activated forms of Src are known to undergo ubiquitination mediated by Cbl, an E3 ubiquitin ligase. COS-1 cells co-transfected with ubiquitin and either wt c-Src or c-SrcY527F showed the presence of an ubiquitin ladder on Src. By contrast, neither c-SrcG2A nor kinase dead cSrc (K295R) were ubiquitinated. Thus, only kinase active forms of Src and not kinase dead or non-myristoylated forms are targeted for degradation. We postulate that myristoylation at the N-terminus contributes to formation of a kinase active conformation of Src that is then degraded via a Cbl mediated pathway. These findings suggest that defects in myristoylation could affect c-Src kinase activity and overall stability by interfering with Cbl/ubiquitin mediated degradation.

2413/B121

Aquaporin-4 Dynamics and Determinants of Assembly in Orthogonal Arrays Revealed by Single-Molecule Fluorescence Imaging.

J. M. Crane, A. S. Verkman; University of California San Francisco, San Francisco, CA

Freeze-fracture electron microscopy (FFEM) indicates that aquaporin-4 (AQP4) water channels can assemble in cell plasma membranes in orthogonal arrays of particles (OAPs). We investigated the determinants and dynamics of AQP4 assembly in OAPs by visualizing fluorescently labeled AQP4 isoforms and mutants in cell membranes using quantum dot single particle tracking and total internal reflection fluorescence microscopy. In several transfected cell types, including primary astrocyte cultures, the long N-terminus 'M1' form of AQP4 diffused freely with diffusion coefficient ~5 x 10^{-10} cm^2/s, covering ~5 μm in 5 min. The short N-terminus 'M23' form of AQP4, which by FFEM was found to form OAPs, was relatively immobile, moving only ~0.4 μm in 5 min. Actin modulation by latrunculin or jasplakinolide did not affect AQP4-M23 diffusion, but deletion of its C-terminal PDZ-binding domain increased its range by ~2-fold over minutes. Biophysical analysis of short-range AQP4-M23 diffusion within OAPs indicated a spring-like potential with a restoring force of ~6.5 pN/μm. Additional experiments indicated that AQP4-M1 and AQP4-M23 isoforms do not co-associate in OAPs, and that OAPs are relatively fixed, non-interconvertible assemblies that do not require cytoskeletal or PDZ-mediated interactions for formation. Analysis of AQP4 deletion mutants revealed progressive disruption of OAPs by addition of 3-7 residues at the AQP4-M23 N-terminus, with polyalanines as effective as native AQP4 fragments. OAPs disappeared upon downstream deletions of AQP4-M23, which, from analysis of point mutants, involves N-terminus interactions of residues Val24, Ala25 and Phe26. AQP1, an AQP4 homolog that does not form OAPs, was able to form OAPs upon replacement of Met1 through Lys8 with Met23 through Thr31 of AQP4. Our results indicate that OAP formation by AQP4-M23 is stabilized by a homo-tetrameric interaction involving hydrophobic N-terminus residues, and that absence of OAPs in AQP4-M1 results from non-selective blocking of this interaction by seven residues just upstream from Met23.

2414/B122

Near-Field Optical Observation of Single Molecule Mobility within a Nuclear Pore.

M. Herrmann[2,3], N. Neuberth[1], J. Wissler[1,3], J. Pérez[1], D. Gradl[2,3], A. Naber[1,3]; [1]Institut für Angewandte Physik, Universität Karlsruhe (TH), Karlsruhe, Germany, [2]Zoologisches Institut II, Universität Karlsruhe (TH), Karlsruhe, Germany, [3]DFG-Center for Functional Nanostructures (CFN), Universität Karlsruhe (TH), Karlsruhe, Germany

A method for applying near-field scanning optical microscopy (NSOM) to freestanding biological membranes in physiological solution has been established. An unfixed membrane spanned over 20-µm-sized holes in a flat surface is imaged at a lateral resolution of < 50 nm by scanning the NSOM probe, a light-emitting nano-aperture, across the unsupported membrane patch. The capabilities of this method are demonstrated by imaging the densely packed pores in manually prepared, freestanding nuclear envelopes (NE) of *Xenopus laevis* oocytes. Nuclear pore complexes (NPCs) were fluorescently double stained with antibodies specific for different nucleoporins and visualized at high optical resolution at different emission wavelengths. The unsupported NE separated two different fluid compartments and thus allowed us to observe fluorescently labeled proteins binding to and passing through the NPCs by placing the near-field probe above a pore and measuring the fluorescence intensity as a function of time. In contrast to other optical super-resolution techniques, NSOM provides an extremely short depth of field in axial direction of ~10-30 nm which enabled us to illuminate only the basket of the NPC. Therefore, the fast diffusional motion of single molecules could be temporally and spatially resolved on a nanoscale for the first time. By using NSOM in combination with fluorescence correlation spectroscopy (FCS), we were able to determine the characteristic diffusion time of the transport factor NTF2 within the transport channel of the nuclear pore complex. Furthermore, we determined the total dwell time of NTF2 in the transport channel and found a good correspondence with results of previous studies based on single molecule tracking techniques. The combination of NSOM and FCS introduced in this paper offers a new way to study fast processes on a nano-scale and might help to understand the translocation through NPCs in more detail.

2415/B123

Yeast Cells Defective in Nuclear Pore Complex Biogenesis and Regulation of Nuclear Envelope Dynamics Carry Mutant Alleles of APQ12 and BRR6.

M. J. Wolyniak[1], J. J. Scarcelli[1], C. A. Hodge[1], V. Choudhary[2], R. Schneiter[2], C. Cole[1]; [1]Biochemistry, Dartmouth Medical School, Hanover, NH, [2]Medicine, University of Fribourg, Fribourg, Switzerland

In all eukaryotes, the nuclear pore complex (NPC) plays a conserved and fundamental role in the transport of macromolecules between the nucleus and cytoplasm. While progress has been made in defining subcomplexes of the NPC, major gaps remain in our understanding of NPC assembly and the membrane fusion events that create the NPC channel. Recent work from our lab identified the integral membrane protein Apq12 as a potential critical link between NPC biogenesis and nuclear membrane dynamics. Cells lacking Apq12 exhibit defects in nuclear envelope (NE) morphology, mislocalization of a subset of cytoplasmic fibril nucleoporin proteins, and cold-sensitive growth. The ability of membrane-fluidizing agents to suppress the *apq12Δ* phenotype suggests that Apq12 plays a role in controlling membrane dynamics. To further explore Apq12's role in NPC assembly, we conducted a high-copy suppressor screen to identify genes that, when over-expressed, would permit growth of *apq12Δ* cells at 16°C. One gene that was identified in the screen was *BRR6*, which encodes an essential NE integral membrane protein. A cold-sensitive *BRR6* allele, *brr6-1* (de Bruyn Kops and Guthrie 2001), shows similar phenotypes to those of *apq12Δ* cells at low temperatures, including altered NPC distribution, nucleoporin mislocalization, and NE morphology. Synthetic lethality occurs when the *brr6-1* mutation is combined with *apq12Δ*. Both *apq12Δ* and *brr6-1* cells have synthetic interactions with mutants affecting multiple lipid biosynthetic pathways, altered response to agents that interfere with normal lipid biosynthesis and regulation, and abnormal sterol profiles. Our findings suggest that Apq12 and Brr6 may play key roles in promoting adjustments to NE lipid composition that are critical to the maintenance of NPC biogenesis in response to environmental changes. A high copy suppression screen is underway using the *brr6-1* allele. Surprisingly, the screen also identified a 771-basepair fragment of *UTP10*, which encodes an essential component of the ribosomal small subunit processome required for 18S rRNA synthesis. The gene fragment represents the HEAT-repeat containing 3' end of Utp10. Full-length *UTP10* was unable to suppress *apq12Δ* cold sensitivity.

2416/B124

Yeast Lipid Particles: Lipidome Meets Proteome.

K. Grillitsch[1], M. Connerth[1], B. Rietschel[2], M. Karas[2], H. Koefeler[3], G. Daum[1]; [1]Institute of Biochemistry, Graz University of Technology, Graz, Austria, [2]Institute of Pharmaceutical Chemistry, Johann Wolfgang Goethe University, Frankfurt, Germany, [3]Center for Medical Research, Medical University of Graz, Graz, Austria

In the yeast *Saccharomyces cerevisiae* as in other eukaryotes neutral lipids are a reservoir of energy and building blocks for membrane lipid synthesis. The yeast neutral lipids, triacylglycerols (TAG) and steryl esters (STE), are stored in so called lipid particles (LP) in a biologically inert form of fatty acids and sterols. Prerequisite for the understanding of LP function and structure is elucidation of their molecular equipment. For this purpose, we performed conventional analysis and mass spectrometric analysis of lipids (TAG, STE, phospholipids), but also of proteins which are present on the surface of LP. These analyses were carried out with LP from cells grown on the two carbon sources glucose and oleic acid. Results obtained by these methods revealed marked differences in the lipidome, but also in the proteome of LP isolated from yeast cells grown under different conditions. Moreover, proteome analysis of LP led to identification of several new putative LP proteins. The detailed analysis of the lipid and protein composition of this organelle will help to deepen our knowledge regarding function of LP in the cellular interplay of organelles. Supported by FWF projects 18857 and W901-B05.

2417/B125

Hph1, a Calcineurin Substrate, Interacts with Components of the ER Protein Translocation Machinery in Yeast.

F. J. Pina[1], A. F. O'Donnell[1], J. P. Miller[2], H. Piao[3], M. S. Cyert[1]; [1]Department of Biology, Stanford University, Stanford, CA, [2]Buck Institute, Novato, CA, [3]Department of Biological Chemistry, UCLA, Los Angeles, CA

Calcineurin (CN), a Ca2+/calmodulin-dependent phosphatase, is critical in Ca2+ signaling and is required for cell survival during environmental stress in *S. cerevisiae*. Hph1, a protein of unknown function, is a CN substrate important for mediating cell stress response. Hph1 shares 23% identity to its homologue, Hph2, and these proteins share overlapping function in regulating cell adaptation to alkaline pH, high salt, cell wall stress, or elevated metal ion concentrations. We have shown that Hph1 and Hph2 interact *In Vivo* and that Hph1, but not Hph2, is a substrate of calcineurin. Hph1 and Hph2 are anchored to the ER membrane by a single transmembrane domain at the C-terminus and localize in a punctate pattern. Both proteins also contain a coiled-coil motif which is required for their function. To better understand the biological function of Hph1 and Hph2 we performed a membrane-based yeast two-hybrid screen. From that screen we identified interactions between Hph1 and Hph2 with several proteins involved in metal ion homeostasis, amino acid permeases, and two components of the ER protein translocation machinery, Sec71 and Sec61. Sec71 encodes one subunit of a complex (also containing Sec72, Sec62 and Sec63), which acts post-translationally to promote translocation into the ER; Sec61 encodes a subunit of the translocation pore. We constructed functional GST-Hph1 and GST-Hph2 fusions and probed their interactions with 6xHIS-HA-ZZ-tagged proteins from the yeast mORF collection. To date we have verified the interaction between Hph1 and Sec71, and have shown that Hph1 interacts with Sec72 and Sec63. Interaction of Hph2 with these proteins is being tested. We also investigated cells lacking both Hph1 and Hph2 for defects in ER protein translocation. The vacuolar proteinase, CPY, and the alpha mating factor precursor both require the Sec71, Sec72, Sec62 and Sec63 complex for efficient translocation; however, no translocation defects for either protein were observed in cells lacking Hph1 and Hph2. We hypothesize that Hph1 and Hph2 regulate the translocation of a specific set of proteins that are required during conditions of environmental stress, and are currently examining candidate proteins.

2418/B126

Reconstitution of a Ternary Complex Containing Oligosaccharyltransferase, Translocon and Ribosome.

Y. Harada, W. Lennarz; Biochemistry and Cell Biology, State University of New York at Stony Brook, Stony Brook, NY

The nascent polypeptide synthesized by ribosomes bound to the endoplasmic reticulum (ER) membrane passes into the lumen through the Sec61 complex (translocon) channel in the membrane. This process is called co-translational translocation. If the polypeptide possesses the *N*-glycosylation sequence (Asn-Xaa-Ser/Thr, Xaa except for Pro), the polypeptide will be co-translationally *N*-glycosylated by the oligosaccharyltransferase (OT) complex on the luminal face of the ER. Three protein complexes, OT, Sec61 complex and ribosomes have been proposed to form the protein synthesizing- and *N*-glycosylating-complex on the ER membrane. Here, we report the reconstitution of an OT-Sec61-ribosome ternary complex in detergent using purified yeast proteins. Ribosomes were incubated with the OT and Sec61 complexes and then analyzed by sucrose gradient ultracentrifugation. The OT and Sec61 complexes were found to co-migrate with the ribosomal fraction as confirmed by measuring A_{260} for ribosomes and by SDS-PAGE/Western blotting for the OT and Sec61 complexes. OT could be recovered from the ribosomal fraction by ConA lectin-beads because it is known to contain *N*-glycosylated subunits. Interestingly, the Sec61 complex and ribosomes were also found in the fraction bound to ConA beads despite the fact that they do not contain *N*-glycosylated proteins as determined by SDS-PAGE/ConA blotting. These findings indicate that the OT, Sec61 complexes and ribosomes form a ternary complex *in vitro*. Furthermore, in both rough ER membranes and puromycin/high salt-treated ER membranes, OT could be cross-linked with the Sec61 complex with a 12 Å bifunctional cross-linking reagent. Therefore, we propose that OT and Sec61 complexes together form a platform for ribosome-mediated protein translocation and *N*-glycosylation on the ER membrane. (Supported by NIH Grant GM 33184)

2419/B127

Bimolecular Complementation Reveals Essential Interactions among Glycoproteins of Herpes Simplex Virus That Are Required to Trigger Cell-Cell Fusion and Virus Entry.

R. J. Eisenberg, D. Atanasiu, E. Lazear, G. H. Cohen; Microbiology, University of Pennsylvania, Philadelphia, PA

Herpes simplex virus entry into cells and cell-cell fusion requires viral glycoproteins gD, gB and a complex of gH/gL. Cell receptor binding by gD triggers steps leading to fusion, an event carried out by gB and gH/gL. The structure of gB reveals that it has the characteristics of a viral fusion protein, but to function in virus-cell or cell-cell fusion, gH/gL must be present in the same membrane. We previously showed that cell-cell fusion is triggered by addition of the gD ectodomain (gD306t) to receptor-bearing cells expressing gB and gH/gL. We used bimolecular complementation (BiMC) to determine whether gD first causes gB to interact directly with gH/gL. Specifically the cleaved N- and C-terminal halves of EYFP were fused to the cytoplasmic tails of gH and gB. Receptor-bearing cells were co-transfected with these plasmids along with gL. Protein-protein interactions between gB and gH/gL were detected by restoration of EYFP fluorescence (BiMC). Prior to addition of gD306t, there was no BiMC or fusion. However, within 10 minutes of gD addition, cells formed syncytia that exhibited EYFP fluorescence. Next we determined whether the gB and gH/gL interaction is necessary for fusion. First, we found that monoclonal antibodies to gB or gH that can neutralize virus infection and block fusion also block BiMC, whereas non-neutralizing antibodies do not. Thus, the interaction between gB and gH/gL is an important event in both virus entry and cell fusion. Second, we found that receptor binding by gD is necessary but not sufficient to trigger BiMC, i.e., gD mutants that fail to bind receptor also fail to trigger BiMC and fusion. We constructed a gD mutant that binds receptor, does not trigger cell-cell fusion, but still induces the gB-gH/gL interaction. Using this mutant, we found that the region of gD mutant necessary for triggering BiMC is distinct from the receptor binding region, thus separating these two functions. These and other data suggest a model whereby gD binds to receptor and undergoes conformationals changes. These changes expose gD residues that induce gB to interact with gH/gL. We suggest that this complex of gB and gH/gL cooperates to carry out cell-cell and virus-cell fusion.

2420/B128

The Role of yeh2 in the Yeast Mating Pathway.

S. A. Jones, G. G. Hutchins; Biology, Fort Lewis College, Durango, CO

The objective of this study is to characterize the potential role of Yeh2 in the *S. cerevisiae* yeast mating pathway. Previous work established Yeh2 as a steryl ester hydrolase in the plasma membrane (JBC 280:13321-13328). Preliminary data for this study demonstrated that Yeh2

binds to the Ste7 MAP kinase kinase of both the mating and filamentation signaling pathways using a yeast 2-hybrid assay. In this current study, the response to mating pheromone in wild type and *yeh2* deletion strains was compared using several assays. The relative activation of MAP kinases Fus3 and Kss1 in response to mating pheromone was measured in the 2 yeast strains using a phosphorylation-specific MAP kinase antibody in an immunoblot. The 2 yeast strains were also compared for G_1 arrest in response to mating pheromone using a halo assay. Gene expression in response to mating pheromone was measured in the 2 yeast strains transformed with plasmid containing the *FUS1-lacZ* reporter. There was not a significant difference in phosphorylation of Fus3 and Kss1 after exposure to mating pheromone in the wild type and *yeh2* deletion strains. Additionally, deletion of the *yeh2* gene did not have a significant effect on G_1 arrest using the halo assay. However, the *yeh2* deletion strain showed significantly less β-galactosidase activity than the wild type strain indicating less activation of *FUS1* gene expression in response to mating pheromone. Therefore, Yeh2 does not appear to be involved in regulating activation of the Fus3 and Kss1 MAP kinases or G_1 arrest after mating pheromone addition, but it does appear to play an important role in controlling the expression of a mating-specific gene. This study points toward a possible function for Yeh2 in regulating the activity of mating pathway gene expression.

ER to Golgi Transport (2421 – 2438)

2421/B129

Selective Export of Human GPI-Anchored Proteins from the Endoplasmic Reticulum.

C. Bonnon, H. Hauri; Biozentrum, University of Basel, Basel, Switzerland

Selective export of transmembrane proteins from the endoplasmic reticulum (ER) relies on recognition of cytosolic-domain-localized transport signals by the Sec24 subunit of the COPII vesicle coat. Mammalian cells express four Sec24 isoforms termed A-D. Glycosylphosphatidylinositol-anchored proteins (GPI-APs) constitute a special class of proteins attached to the membrane via a glycolipid moiety. Since GPI-APs are only luminally exposed, they cannot bind directly to Sec24 for ER-export. They may either interact with transmembrane cargo receptors or use alternative mechanisms, but little is known about the underlying mechanism in higher eukaryotes. Using Sec24 isoform-specific silencing in HeLa cells, we report that ER-to-Golgi transport of the GPI-AP CD59 requires Sec24 with a high preference for Sec24C/D isoforms whereas the transport of the transmembrane protein ERGIC-53 is Sec24C/D-independent. Efficient ER-export of CD59 is also impaired when the recycling transmembrane proteins p23 or p24 (known to form a complex) are silenced. Strikingly, p24 and CD59 share the same Sec24C/D preference for ER export. Co-immunoprecipitation suggests that CD59 interacts with p23 and p24. Floatation gradients indicate co-partitioning of CD59 and p23 into a lipid-raft fraction in which the two proteins also interact. These results suggest that ER-export of GPI-APs in higher eukaryotes is Sec24-isoform-selective and mediated by p24 family members acting as cargo receptors.

2422/B130

Cargo Protein Transmembrane Domain Determines Secretory Transport Rate by Driving Concentration in Export Domains.

A. Dukhovny, Y. Yaffe, K. Hirschberg; Pathology, Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel

The cellular destination of secretory proteins is determined by interactions of their targeting sequences with coat-protein complexes. The secretory proteins' transmembrane domain (TMD) also plays a central role in their transport and targeting. However, a comprehensive model that considers both TMD- and targeting-sequence-mediated transport has not been advanced. We developed a method to quantify the dynamic properties of GFP-VSVG sorting and concentration within ER exit sites (ERESs) in living cells. The sorting process is uncoupled from later translocation steps using microtubule de-polymerization. The dynamics of the ER to ERES redistribution of cargo proteins is quantified in single cells by measuring changes in fluorescence-intensity variance after shift to the permissive temperature. Here, we focused on the secretory transport of the thermo-reversible membrane cargo vesicular stomatitis virus G tsO45 (VSVG), for which both the cytosolic di-acidic motif and TMD are obligatory. A quantitative approach was applied to analyze, in living cells, secretory-transport dynamics, as well as cargo concentration of YFP-tagged VSVG mutants with 1, 3, 5, 7, 8 and 9 amino acids deleted from their TMD. Both the kinetics parameters delineating secretory transport and the extent of cargo concentration in ER exit sites were found to correlate with TMD length. These findings demonstrate that the transport machinery's capacity to concentrate cargo strictly depends on the length of the cargo protein's TMD. Also, our findings point at COPII as the cargo selection machinery and demonstrate that COPI/ARF1 are involved with the maintenance of ERES membrane tubulo-vesicular morphology and are necessary for the TMD dependant cargo concentration.

2423/B131

Coupled Dynamics of Organelle Biogenesis and Vesicular Transport during Cell Cycle.

M. Murata[1], A. Adachi[1], K. Fujiki[1], F. Kano[1,2]; [1]Department of Life Sciences, The University of Tokyo, Tokyo, Japan, [2]PRESTO, JST, Saitama, Japan

Semi-intact cell system is the best experimental system that offers intracellular environment for various assays, and it is, what is called, “cell-type test tube”. Our reconstitution systems, based on semi-intact cells coupled with GFP-visualization techniques, will be use in manipulating intracellular conditions for investigating the dynamics or functions of proteins in single cells under a fluorescence microscope. In this paper, we will show the several our reconstitution systems of organelle dynamics using semi-intact cells. By adding the cytosol from cells in mitosis or interphase into semi-intact cells, we could induce the mitotic Golgi disassembly, the mitotic disruption of endoplasmic reticulum (ER) network and following reformation of the network, the disruption of the ER exit sites, and the transport between ER and Golgi, and elucidated the biochemical requirements for the reconstituted processes. As a result, we found that the activation of cdc2 kinase, at the onset of mitosis, induces the phosphorylation of p47 and results in the disassembly of the ER exit sites, and the disassembly caused the inhibition of anterograde transport of GFP-tagged galactosyltransferase analogue protein (GT-GFP), but its retrograde transport continued. The specific inhibition of anterograde transport would accelerate the Golgi disassembly and the continuing retrograde transport would relocate some Golgi components (lipids and the Golgi enzymes) to the ER network at the early phase of mitosis. Thus, the perturbation of the balance between anterograde and retrograde transport is closely coupled to the morphological changes of the Golgi apparatus. In addition, to apply the semi-

intact cell assay to high throughput screening, we developed the powerful equipment, CellTech Station, for fluorescence-based cell imaging system with intact or semi-intact cell array chip.

2424/B132

The Structures of COPII Coats and Cages.

S. Stagg[1,2], P. LaPointe[4,2], A. Razvi[2], C. Gurkan[3], C. S. Potter[2], B. Carragher[2], W. Balch[2]; [1]Chemistry and Biochemistry, Florida State University, Tallahassee, FL, [2]The Scripps Research Institute, La Jolla, CA, [3]Department of Electron Microscopy and Molecular Pathology, The Cyprus Institute of Neurology and Genetics, Nicosia, Cyprus, [4]University of Alberta, Edmonton, AB, Canada

Coat protein complex II (COPII) vesicles are responsible for packaging and transporting over 10,000 different cargo molecules (about one-third of the eukaryotic genome) of widely varying sizes and shapes from the endoplasmic reticulum (ER) to downstream compartments of the secretory pathway. Proteins involved in generating COPII vesicles include the GTPase Sar1, the cargo adaptor Sec23/24, and the cage scaffold Sec13/31. We previously showed that Sec13/31 can self-assemble into a novel cuboctahedron cage, leading us to suggest that Sec13/31 may form cages of increasing size based on the simple rule that the geometry of the cage is dictated by four Sec13/31 heterotetramers combining to form a vertex. We have now solved the structure of an icosidodecahedral COPII coat assembled from both Sec13/31 and Sec23/24 by cryo-electron microscopy (cryoEM) and single particle reconstruction. This new structure reveals for the first time the structure of the adaptor layer and reveals possible mechanisms for the coordination of adaptor interaction with cage formation and for the collection of cargo of varying size. We fit a crystal structure of the Sec13/31 heterotetramer into the cuboctahedron and icosidodecahedron structures, and this lead us to propose a molecular mechanism by which the COPII system can accommodate cargo of diverse sizes and shapes.

2425/B133

Involvement of a Heterotrimeric G Protein in Traffic-Dependent KDEL-Receptor Signalling.

M. Giannotta[1], P. Teodoro[2], M. Grossi[1], M. Capitani[1], C. Ruggiero[1], A. Luini[1], M. Sallese[1]; [1]Cell Biology and Oncology, Consorzio Mario Negri Sud, Santa Maria Imbaro, Italy, [2]Cell Biology Program, Sloan-Kettering Institute for Cancer Research, Memorial Sloan-Kettering Cancer Center, New York, NY

As with other complex cellular functions, intracellular membrane transport involves the coordinated engagement of a series of organelles and machineries; however, the molecular basis of this coordination is unknown. We have identified an endomembrane-based signalling cascade activated by incoming traffic from the ER to the cis-Golgi. Here, the signal for incoming traffic is provided by chaperones: these reach the cis-Golgi and bind to the KDEL receptor (a seven-transmembrane-domain protein known to cycle between the ER and the Golgi complex to retrieve chaperones that escape from the ER). This in turn triggers a signalling cascade that results in the activation of a pool of Src family kinases (SFKs) located on the Golgi complex. This signalling is crucial for intra-Golgi transport. To investigate the molecular mechanisms through which the KDEL receptor activates these SFKs, we have performed interaction studies using two-hybrid and co-immunoprecipitation assays. Our data show that the KDEL receptor can activate SFKs through a direct interaction, and/or through classical G-protein-dependent signalling. Indeed, we have evidence for the involvement of the Galphaq/11 subunit of heterotrimeric G proteins and its signalling effector Ca2+ in the activation of the SFKs. In summary, we believe that the KDEL receptor behaves as a G-protein-coupled receptor and that Galphaq/11 has a crucial role in the transmission of the signal from the KDEL receptor to the SFKs. This allows the balancing of the membrane input that arrives at the Golgi complex with the signal level generated, so as to maintain the dynamic equilibrium of the Golgi complex.

2426/B134

A High Content, Functional Genomics Approach to the Regulation of Golgi Structure Suggests an Unexpected Mechanism for the Regulation of Glycosylation.

S. Ng, V. Racine, F. Bard; Institute of Molecular and Cell Biology, Singapore, Singapore

The Golgi apparatus in mammalian cells is a highly complex structure. This complexity is mirrored in the glycosylation reactions that yield various and elaborate glycans. It has long been proposed that the precise distribution of glycosylation enzymes in Golgi stacks is critical for the process of glycans building. The molecular mechanisms that generate the Golgi structure are poorly understood at present. To identify what genes are involved in the process, we performed an RNA interference screen based on a high throughput confocal microscopy assay. But if the Golgi presents a high level of complexity at the ultrastructural level, it appears mostly as a compact, perinuclear organelle at the light microscopy level. To supplement the limited resolution of light microscopy and identify more genes that regulate the organization and function of the Golgi, we used a triple labeling of the Golgi with a cis-medial cisternal marker for core Golgi organization, a lectin to monitor glycosylation activity and a trans-Golgi network marker. Interestingly, whereas these Golgi markers are perfectly colocalized in normal cells, perturbation of Golgi dynamics by various treatments lead to a dissociation of the markers and independent variations in their intracellular distribution. Automated image analysis was used to extract multiple numerical features for each markers. Reference phenotypes were used to normalize the numerical features and drive the clustering of experimental phenotypes. After screening for the whole human kinome and phosphatome, we identified several phenotypic clusters. One of these clusters, containing seven kinases and three phosphatases, shows a marked and unexpected increase in lectin labeling intensity, coupled to dispersion in the ER of specific Golgi enzymes. As most of these kinases and phosphatases belong to gene families implicated in signal transduction pathways, our results suggest that specific signal transduction pathways could impact Golgi organization to regulate glycosylation activity.

2427/B135

P28, a Novel ERGIC/Cis-Golgi Protein, Required for Golgi Ribbon Formation.

E. Kögler, C. Bonnon, S. Mitrovic, H. Ben-Tekaya, H. Hauri; Biozentrum, University of Basel, Basel, Switzerland

The mammalian Golgi apparatus consists of up to 7 individual cisternae that are stacked in a polarized manner to form the compact zones of the Golgi. Several stacks are linked into a compact ribbon via highly dynamic lateral bridges in non-compact zones. Little is known about the

proteins involved in maintaining the Golgi structure. Here we report on the characterization of p28, a new γ-subfamily member of the p24 membrane proteins, previously identified as CGI-100 in a proteomic approach of purified ERGIC membranes. P28 localizes to ERGIC and cis-Golgi in human HeLa and HepG2 cells and is enriched in the ERGIC after brefeldin A treatment, indicating that p28 is a cycling protein. Overexpressed p28 accumulates in the ER, but assumes normal localization when p23 is co-transfected, and the two proteins co-immunoprecipitate suggesting some interdependence. Depletion of p28 by siRNA leads to a striking fragmentation of the Golgi without affecting the localization of microtubules, coatomer or tethers such as GM130 and p115. At the ultrastructural level the Golgi fragments can be identified as mini-stacks with apparently unchanged cis-trans morphology, but they are not linked into a ribbon. Fluorescence recovery after photobleaching experiments confirm the lack of linkage of the mini-stacks. Golgi reformation studies reveal that the lack of p28 renders the Golgi incompetent to form lateral connections. We conclude that the formation of a Golgi ribbon requires p28 in addition to tethers, coatomer and SNAREs.

2428/B136

A Polybasic Sequence in the Cytoplasmic Domain of Fcεriγ Regulates Surface Expression and Possibly Stimulated Phosphorylation of the Ige-Receptor.

L. Vasudevan[1], A. Wiesner[2], B. Baird[2,1], D. Holowka[2]; [1]Molecular Biology and Genetics, Cornell University, Ithaca, NY, [2]Chemistry and Chemical Biology, Cornell University, Ithaca, NY

Mast cells mediate allergic responses via the high affinity receptor for immunoglobulin E, FcεRI, that is comprised of a ligand binding α subunit, a tetramembrane spanning β subunit, and a disulfide-bonded γ homodimer. The surface expression and antigen-stimulated phosphorylation of FcεRI are necessary to initiate mast cell signaling and for subsequent release of allergic mediators. To investigate roles for phosphatidylinositol 4,5-bisphosphate ($PI(4,5)P_2$) in regulating FcεRI function, we stably overexpressed murine wt and catalytically inactive mutant type I alpha phosphatidylinositol 4-phosphate 5-kinase (PIP5kinase-Iα), an enzyme that catalyzes the conversion of PI4P to $PI(4,5)P_2$, or Inp51, the $PI(4,5)P_2$-specific 5'-phosphatase from yeast, in RBL-2H3 mast cells. Overexpression of wt PIP5kinase-Iα increased stimulated phosphorylation of FcεRI, whereas, expression of mutant PIP5kinase-Iα or Inp51 reduced this phosphorylation. We found that antigen-stimulated receptor endocytosis was enhanced when Inp51 was expressed suggesting, that PI (4, 5) P2 may regulate receptor phosphorylation by regulating receptor stability at the plasma membrane. To investigate whether a polybasic sequence (RLKIQVRK) in the juxtamembrane region of the cytoplasmic segment of FcεRIγ ($FcεRIγ_{cyt}$) may be involved in mediating electrostatic interactions with $PI(4,5)P_2$ at the plasma membrane, we used a chimeric αγγ receptor containing extracellular domain of FcεRIα subunit fused to the transmembrane and cytoplasmic segments of FcεRIγ subunit. We sequentially mutated the two arginine and two lysine residues to alanine, thereby reducing the net positive charge in this sequence. We found that mutations of three or more basic residues in the polybasic sequence of $FcεRIγ_{cyt}$ caused the retention of mutant αγγ receptors in the endoplasmic reticulum (ER). Furthermore, coexpression of ER-targeted MARCKS effector domain blocked the export of wt αγγ receptors from the ER. Our results reveal an important role for the polybasic sequence of $FcεRIγ_{cyt}$ in receptor export from the ER, and suggest that this sequence may also be involved in the regulation of receptor phosphorylation by stabilization at the cell surface via interactions with $PI(4,5)P_2$.

2429/B137

Reconstitution of Anterograde and Retrograde Transport between the Endoplasmic Reticulum and the Golgi Apparatus in Semi-Intact CHO Cells: the Role of Yip1A in Retrograde Transport.

F. Kano[1,2], S. Yamauchi[1], N. Nakamura[3], M. Murata[1]; [1]Department of Life Sciences, The University of Tokyo, Tokyo, Japan, [2]PRESTO, JST, Saitama, Japan, [3]Division of Life Sciences, Kanazawa University, Kanazawa, Japan

We reconstituted anterograde and retrograde vesicular transport of 1-60 amino acids of galactosyltransferase fused to GFP (GT-GFP), a Golgi marker which recycles between the endoplasmic reticulum (ER) and the Golgi apparatus, in semi-intact Chinese hamster ovary (CHO) cells. CHO-GT cells, which stably express GT-GFP, were permeabilized with streptolysin O, and incubated with L5178Y cytosol and ATP regenerating system at 32 degree. The Golgi or the ER region was bleached by high intensity laser illumination. We then monitored the recovery of fluorescence within the bleached Golgi or the ER region to measure the extent of anterograde or retrograde transport, respectively. Using the assay, we examined the biochemical requirements for the anterograde and retrograde transports of GT-GFP, and confirmed that both transports were dependent on cytosol, the hydrolysis of nucleotides, and microtubule integrity. Recombinant protein of Sar1T39N, a GTP-restricted form of Sar1, which plays an important role in the assembly of COP II coat on the ER membrane, inhibited the anterograde transport in a dose-dependent manner, but did not have an inhibitory effect on retrograde transport. We also investigated the role of Yip1A in anterograde and retrograde transport. Yip1A, a mammalian homologue of yeast Yip1p, is a multi-spanning membrane protein, which localizes to the Golgi and ER exit sites. Although Yip1p is known to be essential for ER-to-Golgi transport in yeast, the precise role of Yip1A in mammalian cells remains unclear. We found that the cytoplasmic N-terminal portion of Yip1A inhibited the retrograde transport of GT-GFP, but not that of p58/ERGIC58, which is a protein marker for ER-Golgi intermediate compartments and cycles between ERGIC and the ER. These results suggested that Yip1A was involved in COPI-independent retrograde transport from the Golgi to the ER.

2430/B138

Luminal Vesicular Calcium Regulates Residual Coat Retention, Fusogenicity and Size of Pre-Golgi Secretory Intermediates.

M. Bentley[1,2], D. C. Nycz[1,2], A. Joglekar[3], J. Hay[1,2]; [1]Division of Biological Sciences, University of Montana, Missoula, MT, [2]Center for Structural & Functional Neuroscience, University of Montana, Missoula, MT, [3]University of Michigan, Ann Arbor, MI

Knowledge of regulatory relationships integrating vesicle coats, tethers and SNAREs is rudimentary. Whether residual vesicle coat proteins remain after tethering to regulate vesicles' fusogenicity is unknown. We discovered an unexpected inhibitory role for luminal Ca2+ released from homotypically fusing COPII vesicles. In vitro COPII vesicle fusion and subsequent cargo mixing was stimulated 3-6 fold by the fast calcium chelator BAPTA, but not by the slower EGTA, despite these chelators' similar equilibrium buffering capacities. The differential affects appeared to be due to differing abilities to rapidly chelate Ca2+ leaking from COPII vesicles. Immunoisolation and immunoblotting of the fusion intermediates established that specific peripheral membrane proteins, including the COPII subunit sec31 and the COPI subunit

beta-COP were released in a BAPTA-specific fashion. Hence, leaking luminal Ca2+ favors the retention of select coat molecules which appears to delay membrane fusion and/or cargo mixing. Apoptosis-linked gene 2 (ALG-2) containing five EF-hand domains was previously implicated in the stabilization of ER exit sites, though a function in vesicle formation was not apparent. Here, addition of purified ALG-2 protein to fusion reactions simultaneously increased COPII retention and inhibited COPII vesicle fusion, suggesting that ALG-2 may represent the Ca2+ sensor for the luminal Ca2+ effects on homotypic fusion. Indeed, ALG-2 mutants incapable of responding to Ca2+ failed to inhibit fusogenicity, and chelation of leaking luminal Ca2+ with BAPTA rendered wildtype ALG-2 unable to inhibit. Our in vitro results indicate that luminal Ca2+ efflux, acting through ALG-2 and coat retention, may temporally regulate COPII vesicle fusion and/or cargo mixing and thus orchestrate orderly VTC biogenesis and maturation. In support of this model, we found that specific depletion of luminal calcium in living NRK cells using the SERCA pump inhibitors thapsigargin or cyclopiazonic acid (CPA) caused a rapid redistribution of cycling intermediate compartment membrane proteins to the periphery and massive expansion of peripheral VTC elements in a manner consistent with excessive or premature COPII vesicle fusion.

2431/B139

Organisation of ER Exit Sites - Localisation of Sec16 to Transitional ER.

H. Hughes[1], K. Schmidt[1], A. Budnik[1], C. Noakes[1,4], A. Johnson[1,5], D. A. Carter[2], P. Verkade[1,2], P. Watson[1,3], D. J. Stephens[1]; [1]Cell Biology Laboritories, Biochemistry, University of Bristol, Bristol, United Kingdom, [2]Wolfson Bioimaging Facility, University of Bristol, Bristol, United Kingdom, [3]Cardiff School of Biosciences, Cardiff University, Cardiff, United Kingdom, [4]Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom, [5]Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

The COPII complex mediates the selective incorporation of secretory cargo and relevant machinery in to nascent budding vesicles at specialized sites on the endoplasmic reticulum membrane called transitional ER. In humans, COPII-coated vesicles bud from these sites and then fuse to form the ER-Golgi intermediate compartment (ERGIC), to which COPI is recruited. Here, we show using light and electron microscopy that, at steady-state, Sec16 localizes to cup-like structures of transitional endoplasmic reticulum that are spatially distinct from the localization of other COPII coat components. We show that Sec16 defines the transitional ER while Sec23-Sec24 and Sec13-Sec31 define later structures that precede but are distinct from the ERGIC. Furthermore, the steady-state localization of Sec16 is independent of the localization of downstream COPII components Sec23-Sec24 and Sec13-Sec31. Sec16 cycles on and off the membrane at a slightly slower rate, and shows a significantly greater immobile fraction, than other COPII components, indicating that it could serve as a platform for assembly of other components.

2432/B140

VLDL Transport Vesicle (VTV) Utilizes Rsec22b as V-SNARE to Fuse with Hepatic Golgi.

S. Siddiqi; Medicine - Gastroenterology, the University of Tennessee Health Science Center, Memphis, TN

The movement of very low-density lipoprotein (VLDL) from the endoplasmic reticulum (ER) to the Golgi is prerequisite for its secretion from hepatocytes. Our studies (Biochem J. 413(2): 333-342; 2008) established that VLDL exits the ER in a specialized vesicle, the VTV. Fusion of vesicles with their target membranes is mediated by specific soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins. SNAREs are membrane bound proteins present on vesicles (v-SNARE) and their target membranes (t-SNARE). We showed earlier that VTV fuses with hepatic Golgi to deliver its cargo, the VLDL, however, SNAREs required for VTV-Golgi fusion are not known. Here we identify a v-SNARE that mediates VTV-Golgi fusion. We separated the VTV and ER membrane proteins by 2D SDS-PAGE. Comparative analysis of 2D-gels suggested 5 proteins were concentrated in VTV compared to the ER. Mr and pI values of these proteins were calculated and submitted to SWISS-PROT. Of 5 spots, the most prominent protein band (Mr 25; pI 8.85) gave several possible proteins, of which VAMP7 and rSec22b were of interest. Mass spectroscopic and immunoblotting data revealed that rSec22b was concentrated in VTV whereas VAMP7 was not identified both in the VTV and hepatic ER. Our results show that rSec22b binds to apolipoproteinB100. We developed an in vitro fusion assay to determine the delivery of VLDL from the VTV to the cis-Golgi lumen. To test the functional role of rSec22b in fusion of VTV with cis-Golgi, we incubated anti-rSec22b antibodies or preimmune IgGs with 3H-TAG-loaded VTVs at 4 oC for 1 hour, the unbound antibodies were removed by washing and treated VTVs were used in fusion assay. Pre-incubation of the VTVs with anti-rSec22b antibodies suppressed the delivery of VTV-3H-TAG to the cis-Golgi suggesting a functional role for rSec22b in fusion of the VTV with hepatic cis-Golgi.

2433/B141

The Amino End of Gint3 Interacts with Rab GDI and Gint3 Localizes to Vesicles.

P. Amin[1], R. Ayabe[1], A. Nordmann[1], S. Li[1], C. Cheng[1], R. Rawle[2], M. Johal[2], C. Cheney[1]; [1]Biology, Pomona College, Claremont, CA, [2]Chemistry, Pomona College, Claremont, CA

A major role of rab GDP dissociation inhibitor (GDI) is to recycle rab GTPases from target membranes to donor membranes. The removal of rab from membranes and insertion of the geranylgeranyl group of rab into the lipid-binding pocket of GDI likely requires cofactors, such as the chaperone hsp90 (Chen and Balch, 2006). Previously, our lab has identified Drosophila Gint3 as a GDI interacting protein, using yeast two hybrid interaction. Gint3 is a protein of unknown function that contains PUG and UBX domains. We have found that His and Flag-tagged GDI, expressed in flies, interacts with Gint3. Further, the amino end of Gint3 interacts with GDI, and not the PUG or UBX domains that are in Gint3. The interaction of Gint3 and amino-Gint3 with GDI was confirmed in the quartz crystal microbalance with dissipation (QCM-D). GFP-tagged Gint3 was seen in puncta in larval hemocytes, which are likely vesicles. Staining of hemocytes with an antibody specific to the amino end of Gint3 showed a similar pattern. We propose that Gint3 associates with vesicles near rabs and facilitates rab extraction from membranes by GDI. Supported in part by NSF RUI grant 0212730 to CMC.

2434/B142

Exp1p: a Novel Adaptor Involved in Trafficking of Membrane Proteins from the ER.

D. L. Morse, M. Crotwell Kirtley, C. Kaiser; Department of Biology, Massachusetts Institute of Technology, Cambridge, MA

Proteins destined for export from the ER are separated from ER resident proteins by packaging into COPII coated vesicles. Our lab previously showed that in *S. cerevisiae* the plasma membrane H+ ATPase (Pma1p) is primarily exported from the ER via COPII coated vesicles containing the Sec24p homolog Lst1p. An *lst1Δ* is viable but exhibits sensitivity to growth in acidic medium due to a decrease in Pma1p at the plasma membrane. We have identified a novel gene, named *EXP1* (ER export of Pma1p), that when overexpressed restores Pma1p plasma membrane localization in an *lst1Δ*. A deletion of *EXP1* is viable, but an *lst1Δ exp1Δ* double mutant is inviable and displays a severe Pma1p trafficking defect. Based on these findings we propose that there are two parallel pathways for Pma1p export from the ER: one mediated by Lst1p and another that requires Exp1p and Sec24p. *EXP1* encodes a 17 kD integral membrane protein, with one N-terminal transmembrane domain and a highly charged cytosolic C terminus. Exp1p fractionates primarily with the ER in wild type cells and with the Golgi in COPI mutant cells indicating that Exp1p cycles between the ER and Golgi. Recombinant Exp1p binds to Sec23/24p in vitro. We have identified short cis-acting sequences in the cytosolic C terminus of Exp1p that are required for ER export and interaction with Sec23/24p. Thus, Exp1p appears to be required for transport of Pma1p from the ER via a Sec24p-mediated pathway. Exp1p represents a novel class of cargo adaptors that expand the cargo loaded into Sec24p-coated vesicles to include specific membrane proteins.

2435/B143

Orm1 and Orm2 Are Members of a New Family of ER Membrane Proteins Involved in Protein Quality Control.

S. Han, A. Chang; Department of Molecular, Cellular & Developmental Biology, University of Michigan, Ann Arbor, MI

Orm1 and Orm2 belong to the conserved ORMDL family of endoplasmic reticulum (ER) membrane proteins in eukaryotic cells. In yeast the two proteins have overlapping function and ~70% amino acid identity with each other. We find that protein quality control is impaired in the absence of Orm1 and Orm2: cell growth is slowed and the unfolded protein response (UPR) is constitutively activated. Moreover, stress resistance is defective so that orm1 orm2 cells are cold-sensitive and have increased sensitivity to agents that increase protein misfolding in the ER, i.e., tunicamycin and the misfolded protein CPY*. Further supporting a role in ER quality control and stress response, Orm2 protein is up-regulated when protein misfolding is increased. In orm1 orm2 cells, there is slowed ER-to-Golgi transport of several secretory pathway cargo (CPY, Gas1, proteinase A). Strikingly, tunicamycin sensitivity and ER-to-Golgi transport in orm1 orm2 cells are suppressed at increased temperature (37°C). We tested whether the suppressing effect of 37°C incubation is mediated by HSR. Surprisingly, suppression of orm1 orm2 cells was not observed when constitutively active Hsf1 was used to induce HSR in the absence of shift to high temperature. We considered the possibility that increased membrane fluidity can account for the ameliorative effects of 37°C. Several high throughput studies have suggested genetic and physical interactions between Orm1, Orm2 and components of the sphingolipid and ergosterol biosynthesis pathways. Because these lipids are thought to promote decreased membrane fluidity, we tested the possibility that reduced sphingolipid synthesis may suppress orm1 orm2 cells. Indeed, suppression was observed upon inhibition of sphingolipid synthesis by the inhibitor myriocin, or by mutation of LCB3 or SUR2, encoding enzymes regulating production of sphingolipid precursor metabolites. We propose a role for Orm1 and Orm2 in maintaining ER quality control by balancing protein and lipid biogenesis.

2436/B144

Molecular Analysis of Trafficking of the Giant Cargo: Type I Procollagen from the Endoplasmic Reticulum.

K. Bajaj, R. Schekman; Dept of MCB, UC-Berkeley, Berkeley, CA

Type I collagen is the major component of fibrils in tendon, bone and skin. Collagen is synthesized as Type I procollagen (PC-1) in a precursor that is co-translationally translocated into the endoplasmic reticulum (ER). Most perinatal lethal cases of Osteogenesis Imperfecta (OI) are caused by mutations in PC-1 that yield improperly folded PC-1 molecules which are retained and degraded in the ER. The mechanism by which 300nm long soluble fibril of PC-1 destined for secretion from the cell is transported from the ER through the Golgi to the plasma membrane has long been a challenge to cell biologists. In an attempt to get insights into the fundamental problem of trafficking of large secretory soluble molecules, we are investigating the PC-1 transport from the ER using permeabilized human fibroblasts cells and an *in vitro* vesicle budding reaction. We have established the *in vitro* budding of Type I procollagen from IMR-90 fibroblasts using rat liver cytosol and purified recombinant human COPII proteins. Packaging of PC-1 in these vesicles is dependent on energy source in the form of ATP and GTP, as required for the transport of ERGIC-53 (ER-Golgi recycling protein)and is inhibited by the non-hydrolyzable analog of GTP (GTPγS).Furthermore this transport is sensitive to the dominant negative H79G mutant of Sar1 protein, suggesting that COPII components are required for PC-1 budding. Moreover, PC-1 in these vesicles results in a fragment resistant to chymotrypsin/trypsin degradation corresponding to the molecular weight of mature Type I collagen, indicating the correctly folded trimeric state of PC-1 in these budded vesicles. Using purified human COPII proteins, we will attempt to establish the minimum requirements for packaging of folded procollagen and compare and contrast these requirements with other reporters of cargo packaging into standard (70 - 80 nm) COPII vesicles.

2437/B145

The Specificity of COPII in the Transport of Vangl2 for Neural Tube Development.

D. Jensen[1], J. Merte[2], D. Ginty[2], R. Schekman[1]; [1]Molecular and Cell Biology, HHMI, University of California - Berkeley, Berkeley, CA, [2]Neuroscience, HHMI, Johns Hopkins University Medical School, Baltimore, MD

The objective of this study was to determine the role of a specific COPII component, Sec24B, in the development of the neural tube. A prior murine forward genetics screen for neural tube defects found a premature stop codon in this protein, a component of the COPII budding complex responsible for the transport of cargo proteins out of the endoplasmic reticulum (ER). Using immunoprecipitation and localization, we have shown that this premature stop codon at Tyr653 renders Sec24B non-functional. Because neural tube development depends on planar cell polarity, we set out to identify what membrane components of the planar cell polarity pathway might depend on Sec24B for their exit from the ER. Through the use of an in vitro COPII budding reaction, we were able to demonstrate that the ER export of Vangl2 is specifically dependent on Sec24B. In addition, we have determined that commonly studied "Looptail" point mutations in the C-terminal cytoplasmic domain of Vangl2 block its exit from the ER in vivo and in vitro. We conclude that Sec24B has a unique role among its paralogs in the ability to transport specific cargo molecules, like Vangl2, and that the efficient ER export of membrane proteins implicated in planar cell polarity is required for proper neural tube development.

2438/B146

Rab2 Recruited Atypical PKC Preferentially Binds to Tyrosinated Alpha Tubulin and Plays a Role in Dynein Association with VTCs.

E. Tisdale, F. Azizi, C. Artalejo; Dept. of Pharmacology, Wayne State University School of Medicine, Detroit, MI

The small GTPase Rab2 requires glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and atypical PKC (aPKC) for retrograde vesicle formation from vesicular tubular clusters (VTCs) that sort cargo to the anterograde pathway from recycling proteins and trafficking machinery retrieved to the endoplasmic reticulum. However, the precise role of GAPDH and aPKC in the early secretory pathway is unclear. GAPDH and aPKC associate directly and independently with microtubules (MTs). MTs undergo a variety of post-translational modifications that influence interactions with effectors and motors. Therefore, a quantitative membrane binding assay was used to characterize the distribution of tyrosinated/detyrosinated alpha tubulin that was recruited by Rab2. We found that Rab2 predominantly recruited tyrosinated alpha tubulin (Tyr-tubulin). However, aPKC was the limiting and essential factor for Tyr-tubulin binding to membranes. Since tyrosination - detyrosination of alpha tubulin influences MT motor protein binding, we determined if Rab2 recruited kinesin or dynein to VTCs. We did not detect any Rab2 recruited kinesin on membranes. In contrast, Rab2 promoted a dose dependent increase in membrane associated dynein that was aPKC-dependent. Based on our combined results, we suggest a mechanism by which Rab2 controls MT and motor recruitment to the VTCs

Trafficking in Polarized Cells (2439 – 2463)

2439/B147

Retinyl Ester Homeostasis in the Adipose Differentiation-Related Protein Deficient Retina.

Y. Imanishi[1], W. Sun[1], T. Maeda[2,1], A. Maeda[1], K. Palczewski[1]; [1]Department of Pharmacology, Case Western Reserve University, Cleveland, OH, [2]Department of Ophthalmology, Case Western Reserve University, Cleveland, OH

The first step in vision is initiated when light strikes retinal photoreceptor cells causing photoisomerization of the rhodopsin chromophore 11-*cis*-retinal to all-*trans*-retinal. The regeneration of rhodopsin requires formation of 11-*cis*-retinal from all-*trans*-retinyl esters in the adjacent retinal pigment epithelium (RPE). Using the intrinsic fluorescence of all-*trans*-retinyl esters, noninvasive two-photon microscopy revealed novel structures, retinyl ester storage structures (RESTs, also called retinosomes), in the RPE. These structures form autonomous all-*trans*-retinyl ester-rich intracellular compartments distinct from other organelles and colocalize with adipose differentiation-related protein (Adfp). To study the function of Adfp, we generated mice lacking exons 2 and 3 of the gene encoding adipose differentiation-related protein, a structural component of RESTs. We found that dark adaptation was slower in $Adfp^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ than in $Adfp^{+/+}$ mice, and that $Adfp^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice had consistently delayed clearances of all-*trans*-retinal and all-*trans*-retinol from rod photoreceptor cells. Two-photon microscopy revealed aberrant trafficking of all-*trans*-retinyl esters in the RPE of $Adfp^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ mice, a problem caused by abnormal maintenance of RESTs in the dark adapted state. Retinyl ester accumulation also was reduced in $Adfp^{\Delta 2\text{-}3/\Delta 2\text{-}3}$ as compared with $Adfp^{+/+}$ mice. These observations suggest that Adfp plays a unique role in vision by maintaining proper storage and trafficking of retinoids within the eye.

2440/B148

The Outer Segment Serves as a Default Destination for the Trafficking of Membrane Proteins in Photoreceptors.

S. Baker[1], M. Haeri[2], P. Yoo[1], S. M. Gospe[1], N. Skiba[1], B. E. Knox[2], V. Arshavsky[1]; [1]Albert Eye Research Institute, Duke University Medical Center, Durham, NC, [2]Biochemistry, Molecular Biology and Ophthalmology, SUNY Upstate Medical University, Syracuse, NY

Photoreceptors are compartmentalized neurons in which all proteins responsible for evoking visual signals are confined to the outer segment. However, the mechanisms responsible for establishing and maintaining photoreceptor compartmentalization are poorly understood. The objective of this study was to investigate the targeting of two related membrane proteins. One protein, R9AP, resides primarily in the outer segment, whereas the second protein, syntaxin 3, is excluded from this compartment. Our approach consisted of generating GFP-tagged or Myc-tagged protein constructs which were then expressed in the photoreceptors of transgenic *Xenopus laevis*. The subcellular localization of these proteins was then assessed by confocal microscopy. Surprisingly, we have found that only syntaxin 3 has targeting information encoded in its sequence and the removal of this targeting information redirects the protein to the outer segment. Furthermore, proteins residing in the endoplasmic reticulum and mitochondria were similarly redirected to the outer segment after removing their targeting signals. In conclusion, these data reveal a pattern where membrane proteins lacking specific targeting information are delivered to the outer segment, which is likely to reflect the enormous appetite of this organelle for new material necessitated by its constant renewal. This also implies that every protein residing outside the outer segment must have a means to avoid this "default" trafficking flow.

2441/B149

Role of Ankyrin G in the Restriction of Sodium Channel Diffusion in Neurons.

A. Brachet[1,2], C. Leterrier[1,2], A. Brechet[1,2], D. Choquet[3], B. Dargent[1,2]; [1]Institut Jean Roche, INSERM UMR641, Marseille, France, [2]Université de la Méditerranée, Marseille, France, [3]Institut Magendie, CNRS UMR 5091, Bordeaux, France

The axonal initial segment (AIS) is a highly specialized sub-domain of neurons where the initiation of the action potential takes place. It also plays a crucial role in maintaining neuronal polarity, as it forms a diffusion barrier that restricts the surface mobility of membrane proteins and phospholipids (Winckler B. et al., Nature. 1999 Feb 25; 397 (6721): 698-701; Nakada C. et al., Nat Cell Biol. 2003 Jul; 5 (7): 626-32). The formation of this barrier is linked to the accumulation of sodium channels and ankyrin G. It was suggested that membrane proteins (sodium channel and adhesion proteins), anchored to the dense submembrane cytoskeleton, function as rows of pickets, which stop the diffusion of all membrane constituents. Genetic, biochemical and cell biology studies converge to a crucial role of ankyrin G, as the key player in organizing the AIS. To study the role of ankyrin G in the restriction of sodium channel mobility, and therefore in the formation of the AIS diffusion barrier, we used a single particle tracking strategy. Difficulties in expressing full Nav1.2 led us to generate chimeric ion channel Kv2.1-Nav1.2 as a suitable tool. The Nav1.2 fragment bears the ankyrin binding motif of sodium channels (Garrido JJ. et al.,

Science. 2003 Jun 27; 300 (5628): 2091-4). We observed that the lateral diffusion of Kv2.1-Nav1.2 is highly restricted at the AIS of mature cultured hippocampal neurons. We determined the critical residues involved in sodium channel-ankyrin G interaction and their effect on Kv1.2-Nav1.2 diffusion. We then studied Kv2.1-Nav1.2 diffusion in young neurons, where no barrier is yet form. In these conditions Kv2.1-Nav1.2 diffusion is also severely restricted at the AIS, pointing to the crucial role of ankyrin G in restricting sodium channel diffusion. To further precise the role of ankyrin G, we conducted experiments in neuroblastoma N2A cells which lack ankyrin G expression. We observed that coexpression of ankyrin G robustly decreased the mobility of Kv2.1-Nav1.2, but had no effect on the diffusion of a construct lacking the ankyrin binding motif. Thus, ankyrin G interaction is able to specifically decrease sodium channel diffusion.

2442/B150

Casein Kinase 2 Directs Sodium Channel Clustering in Axonal Membranes by Regulating Interactions with Ankyrin G.

C. Leterrier[1,2], A. Bréchet[1,2], M. Fache[1,2], G. Ferracci[4,2], A. Brachet[1,2], A. Baude[3,2], M. Irondelle[1,2], S. Perreira[1,2], B. Dargent[1,2]; [1]INSERM UMR641, Marseille, France, [2]IFR11, Université de la Méditerranée, Faculté de Médecine Secteur-Nord, Marseille, France, [3]CNRS UMR6231, Marseille, France, [4]Centre d'Analyse Protéomique, Marseille, France

In neurons, the generation and propagation of action potentials require the precise accumulation of sodium channels at the axonal initial segment (AIS) and in the nodes of Ranvier, two highly specialized sub-domains that assemble through ankyrin G scaffolding. In the present study, we provide evidence for a novel mechanism that specifies sodium channel accumulation in these ankyrin G-containing sub-domains. The ankyrin-binding motif of Nav1.2 channel that determines proper channel targeting and clustering at the AIS requires the presence of a glutamate residue at position 1111, but also several serine residues (S1112, S1124 and S1126). We show that phosphorylation of these serine residues by casein kinase 2 (CK2) regulates Nav1.2 channel interaction with the membrane-binding domain of ankyrins. Furthermore the alpha subunit of CK2 was found to be highly enriched at the AIS and the nodes of Ranvier, both in vivo and in cultured hippocampal neurons. Finally, expression of an ion channel chimera containing the Nav1.2 ankyrin-binding motif perturbed endogenous sodium channel accumulation at the AIS, whereas chimeras containing phosphorylation-deficient motifs did not. Altogether, our findings indicate that the local concentration of CK2 at the AIS and the nodes of Ranvier contributes to the specific accumulation of sodium channels by regulating their interaction with ankyrin G.

2443/B151

Endocytic Trafficking of TrkB Receptor.

C. Proenca[1, 2], F. S. Lee[1, 3]; [1]Psychiatry, Weill Cornell Medical College, New York, NY, [2]Instituto Gulbenkian de Ciencia, Oeiras, Portugal, [3]Pharmacology, Weill Cornell Medical College, New York, NY

Neurotrophins are a small family of closely related polypeptides that influences the growth, differentiation and survival of neurons. These functions are mediated through activation of the Trk tyrosin kinase neurotrohphin receptor. While there has been significant advances in delineating the downstream signal transduction events in the neurotrophin system, less has been established about the sequence of events involved in activated neurotrophin receptor trafficking. Brain derived neurotrophic factor (BDNF) and Neurotrophin 4 (NT-4), both mediate their trophic actions through TrkB tyrosin kinase neurotrophin receptor. However, even though both BDNF and NT-4 activate TrkB receptor with similar affinity, several lines of evidence suggest a difference in their biological output. TrkB receptor has been shown to undergo rapid endocytosis upon activation by BDNF, nevertheless, the subsequent trafficking steps are less clear. With the current study, we investigate the difference in the endocytic trafficking of TrkB receptor and sorting to either: degradation, recycling, or retrograde transport pathways, upon activation by BDNF or NT-4. We also analyzed downstream interacting proteins that might lead to a different distribution of TrkB receptor upon internalization. We analyzed the rates of ligand-dependent degradation and ubiquitination. Our studies indicate that NT-4 induces decreased degradation of TrkB receptor in cultured cortical neurons compared to BDNF, which suggests a differential regulation of the endocytic trafficking of TrkB, mediated by the ligand. Also, we found that NT-4 induces less efficient ubiquitination of TrkB. Moreover, our results suggest that signaling molecules, involved in major biological processes, are differentially regulated by BDNF or NT-4. Our studies are of crucial significance in order to understand how the same neurotrophic receptor can mediate different biological outputs when activated by different neurotrophins.

2444/B152

Dynein Light Chain LC8 Regulates Syntaphilin-Mediated Docking of Axonal Mitochondria.

Y. Chen, C. Gerwin, J. Kang, Z. Sheng; Synaptic Function Section, NINDS, NIH, Bethesda, MD

Mitochondria in the cell bodies of neurons are transported down neuronal processes in response to changes in the local energy and metabolic state. Because of their extreme polarity, neurons require specialized mechanisms to regulate the transport and retention of mitochondria at specific subcellular locations. While approximately one-third of axonal mitochondria are mobile in mature neurons, a large proportion remains in a prolonged stationary phase. Our previous studies using syntaphilin (SNPH) knockout mice in combination with cell biological techniques provided evidence that axon-targeted SNPH can dock mitochondria through its interaction with microtubules (Kang et al., Cell 2008). However, the molecular mechanisms underlying the regulation of SNPH-mediated control of axonal mitochondrial motility remain elusive. In current study, by applying proteomic approach and GST-pull down assays, we reveal that dynein light chain LC8 directly interacts with SNPH both in vitro and in brain homogenates. By mutagenesis and co-immunoprecipitation, we map the LC8 binding motif on SNPH. Both proteins are co-localized along axonal mitochondria when co-expressed in hippocampal neuron, and such co-localization was disrupted when expressing SNPH mutant lacking the LC8 binding sequence. Furthermore, deletion of the LC8-binding sequence reduces the role of SNPH in the immobilization of axonal mitochondria. In contrast, transient expression of LC8 significantly reduces mobility of axonal mitochondria in wild-type but not in SNPH deficient neurons. These results suggest that LC8 can regulate mitochondrial motility though its interaction with SNPH. Study of the mechanistic insights into this regulatory pathway is underway. Future investigations using the snph knockout mice model will provide molecular and cellular details on how LC8-SNPH complex regulates mitochondrial motility and neuronal function and demonstrate whether the snph mice are more susceptible to agents that cause motor neuron degeneration. (Supported by the NINDS Competitive Fellowship Award and the NINDS Intramural Program).

2445/B153

Maturation of rab27b-Coated Secretory Vesicles Involves Association with Both Actin and Microtubule Components in Lacrimal Gland Acinar Cells.

L. Chiang[1], R. Marchelleta[1], F. Yarber[1], S. Karvar[2], S. Hamm-Alvarez[1,3]; [1]Pharmacology and Pharmaceutical Sciences, University of Southern California, Los Angeles, CA, [2]Keck School of Medicine, University of Southern California, Los Angeles, CA, [3]Physiology and Biophysics, University of Southern California, Los Angeles, CA

The purpose of this study was to characterize the maturation process of rab27-coated secretory vesicles (SVs) in acinar epithelial cells by determining their association with cytoskeletal components. Endogenous and adenoviral-mediated rab27b wild type (WT) and mutant (CA, constitutively active; DN, dominant-negative) protein expression and co-localizations with myosin 5c (M5C) and p150^{Glued} (p150) were detected by confocal fluorescence microscopy in primary cultured rabbit lacrimal gland acinar cells (LGAC). Kinetics of rab27b-labeled SV movement were followed by z-stack live-cell confocal microscopy in LGAC after 60m ±33µM nocodazole (Noc) at rest, with carbachol (100µM CCH, 15m), and at 60m after CCH washout. In the lacrimal gland, rab27b, M5C, and the cytoplasmic dynein subunit, p150, are endogenously expressed. Analysis of immunofluorescence labeling patterns suggested that rab27b-labeled SV were co-localized with M5C beneath the apical membrane, particularly on the apical-facing side of the SV. Rab27b also co-localized with p150. Analysis of M5C distribution in LGAC expressing CA and DN rab27b showed changes in the extent of subapical localization. Live cell imaging after stimulation and washout of CCH, to deplete existing SV, was completed in ±Noc LGAC in order to determine whether microtubule depolymerization affected the maturation of newly formed rab27b-coated SVs. Prior to CCH addition, 3D reconstructions suggest similar subapical localization of 1-2µm diameter SVs ±Noc. However, after CCH washout, +Noc LGAC revealed smaller SV which appeared to be attached to basal organelles, compared to more apically localized SVs in -Noc cells. Conclusion: Rab27b-coated SVs associate with M5C and p150; biased apical staining may indicate a mechanism for polarized SV transport on actin. The inability of +Noc LGAC to recover subapically localized rab27b-coated SVs after depletion by CCH stimulation suggests that intact microtubules are necessary for transport of nascent rab27b-coated SVs to the apical region. For the first time in acinar epithelial cells, we have identified a regulatory protein, rab27b, which links together two spatially and maturity distinct stages of SV transport. [NIH EY011386]

2446/B154

Polymeric IgA Receptor Knockout Mice Exhibit Significant Changes in Lacrimal Gland Morphology and Tear Fluid Composition.

M. C. Edman[1], M. Pidgeon[2], C. Okamoto[1], J. E. Schechter[2], S. Hamm-Alvarez[1]; [1]Department of Pharmacology and Pharmaceutical Sciences, University of Southern California, School of Pharmacy, Los Angeles, CA, [2]Department of Cell and Neurobiology, University of Southern California, Keck School of Medicine, Los Angeles, CA

The polymeric IgA Receptor (pIgR) is responsible for the transcytosis of dimeric IgA and the release of secretory IgA and secretory component from mucosal epithelia and exocrine glands such as the lacrimal gland (LG). In coherence with our studies of transcytotic pathways within the LG it was of interest to investigate if pIgR is essential for the maintenance of LG functions by examining tissue morphology and the proteomic profile of the tear film in mice with and without pIgR. LGs were isolated from male 6 or 12-weeks old pIgR knock out (pIgR-/-) and wild type C57/BL6 (WT) mice for histological evaluation by light and electron microscopy (EM). Tear fluid was collected from the same groups of mice after topical stimulation with the acetylcholine analogue carbachol (50 µM) and used for comparison of the proteomic profile between the pIgR-/- and WT mice through 2-D fluorescence difference gel electrophoresis (2-D DIGE). Proteins with a significant abundance difference were identified by Maldi-TOF MS. The 2D-DIGE analysis showed that the abundance ratio of four proteins, including lactoperoxidase, was significantly lower in the 6-weeks old pIgR-/- versus WT mice LG and eleven proteins including lipocortin and secreted carbonic anhydrase (CAR VI) had a higher abundance ratio. In 12-week old mice there were seven proteins with a lower abundance ratio, including albumin and transferrin, while only CAR VI had a higher abundance ratio. At the light microscopy level, no apparent morphological difference between pIgR -/- and WT LG was detected. However, evaluation by EM showed that a large number of the lacrimal acinar cells had altered cell features, which included heavily vesiculated ER and a reduced number of secretory vesicles. The results of this study shows that the absence of pIgR results in altered LG subcellular morphology, including changes in the secretory pathway as well as changes in the abundance of some of the major tear fluid proteins, thus indicating that expression of pIgR is essential for normal lacrimal secretory functions. These findings are consistent with our previous finding that pIgR associates directly with the secretory vesicle protein, rab3D. Support: NIH EY016985 (S.H.A), EY10550 (J.E.S.)

2447/B155

Ultrastructural Transformation of Gastric Parietal Cells Reverting from the Active to the Resting State of Acid Secretion Revealed in Isolated Rat Gastric Mucosa Model In Vitro Processed by Cryotechnique.

A. Sawaguchi, F. Aoyama, S. Ide, T. Suganuma; Anatomy, University of Miyazaki, Faculty of Medicine, Miyazaki, Japan

To elucidate a functional transformation of gastric parietal cells, we have newly developed an isolated rat gastric mucosa model whose parietal cells exhibited a reverting process from the active to resting state of acid secretion. Briefly, the parietal cells were treated with cimetidine following prior stimulation of acid secretion in the model, and cryofixed by plunge freezing for light microscopy or high-pressure freezing for electron microscopy. As a result, immunohistochemistry of H+/K+-ATPase demonstrated a progressive translocation of H+/K+-ATPase from the apical to the cytoplasmic region. The ultrastructure of parietal cells at 5 min in the reverting phase was quite similar to that of maximally stimulated one. However, the apical microvilli of intracellular canaliculi changed bulbous by degrees, resulted in complete occlusion of IC at 60 min in the reverting phase. Corresponding to the ultrastructural changes of microvilli, the immunohistochemistry of ezrin showed a dissociation of ezrin from the apical region at 30 min in the reverting phase. The apical membranes were subsequently internalized into the cytoplasm forming unique penta-laminar membranes. Interestingly, at 90 min in the reverting phase, the penta-laminar membranes formed a number of multilamellar autophagosomes that were intensely labeled for H+/K+-ATPase. Then, the parietal cells exhibited well-developed Golgi apparatus and lysosomal compartments involving the multilamellar membranes at 105 min, and mostly reverted to their resting conformation at 120 min in the reverting phase. The present findings provide new insights into the functional transformation in gastric parietal cells reverting to their resting conformation.

2448/B156

Regulation of the hDlg/hScrib/Hugl-1 Tumour Suppressor Complex.

P. Massimi, M. Thomas, L. Banks; ICGEB, Trieste, Italy

The proper function of the Scribble tumour suppressor complex is dependent upon the correct localisation of its components. Previously we observed dynamic re-localisation of the hDlg component under conditions of osmotic stress. We now show that the other two components of the complex, hScrib and Hugl-1 display similar patterns of expression. This was done using shRNA ablation, immunofluorescence and immunoprecipitation techniques.We demonstrate, by shRNA ablation of hScrib expression, that hDlg and Hugl-1 are in part dependent upon hScrib for their correct localization. Likewise, shRNA ablation of hDlg expression results in aberrant expression of hScrib. However under conditions of osmotic stress this apparent dependency no longer exists: hDlg, hScrib and Hugl-1 localise to cell membranes independently of each other. We also demonstrate an interaction between the three components of the hScrib complex and the t-SNARE syntaxin 4, and show that correct localization of the Scrib complex is in part t-SNARE dependent. This is the first detailed analysis of the co-localisation and function of the hScrib complex in mammalian cells and demonstrates a direct link between the control of the hScrib complex and vesicle transport pathways.

2449/B157

Naked2 Selectively Binds Phosphoinositide PI4,5P2 in a pH-dependent Manner, a Requirement for Naked2 Vesicle Delivery to the Plasma Membrane.

C. Li[1], Z. Cao[1], W. Ding[2], T. Hu[2], R. Deal[1], A. H. Brown[4], R. Coffey[1,2,3]; [1]Departments of Medicine, Vanderbilt University, Nashville, TN, [2]Cell & Development Biology, Vanderbilt University, Nashville, TN, [3]Department of Veterans Affairs Medical Center, Vanderbilt University, Nashville, TN, [4]Department of Pharmacology, Vanderbilt University, Nashville, TN

Naked2 acts as a multi-functional cargo recognition and targeting protein to enable transport of TGFα-containing vesicles to the plasma membrane. We recently identified a basolateral targeting (BLT) domain that ensures proper delivery of TGFα-containing exocytic vesicles to the lower lateral membrane of polarized MDCK cells (Mol Biol Cell 18:3081-3093, 2007). However, the mechanism for Naked2 binding to the plasma membrane and vesicles is unknown. We present evidence that wildtype Naked2 selectively binds phosphoinositide PI4,5P2 in a pH-dependent manner. The binding intensity is strongest at pH7.5 and falls off at pH5.0. A PI4 kinase inhibitor, but not PI3 kinase inhibitors, interrupted Naked2 trafficking. In addition, the N-terminal 36 residues of Naked2 act as a general vesicle recognition domain. A Naked2 N1-36-GFP chimera decorates the plasma membrane and vesicles. Selectivity for TGFα-containing vesicles is conferred by the TGFα tail binding (TTB) domain. The N-terminal 36 amino acids of Naked2 in conjunction with either the myristoyl group or TTB domain localize to vesicles. Thus, the N-terminal 36 residues in the absence of N-myristate may act as a distinct vesicle recognition (VR) domain. Coordinated cooperativity among distinct domains (VR, BLT and TTB) of Naked2 and ion concentration of distinct subcellular compartments confer specific vesicle recognition, directional targeting and cell surface fusion.

2450/B158

The Clathrin Adaptors AP1a and AP1B Function as Non-Equal Partners in Basolateral Polarity.

D. Gravotta, S. Deborde, E. Rodriguez-Boulan; Ophthalmology, Dyson Vision Research Institute Weill Cornell Medical College, New York, NY

We recently reported a new role of clathrin, the regulation of basolateral epithelial polarity and predicted a broad involvement of clathrin adaptors in this process (1). However, to date only the heterotetrameric clathrin adaptor AP1B is known to participate in basolateral protein transport (2). Here, we demonstrate that the highly related adaptor AP1A, which shares with AP1B three subunits and differs only in its medium subunit (μ1A instead of μ1B), performs basolateral transport functions that are distinct from and complementary to those performed by AP1B. Single AP1A knock-down depolarized no basolateral proteins whereas single AP1B knockdown depolarized only a subset of them. Strikingly, dual knockdown caused a dramatic loss of polarity of most basolateral PM proteins. Biochemical assays showed that this resulted from defective intracellular sorting and non polarized delivery of cargo proteins to apical and basolateral PM. Live imaging experiments demonstrated that AP1B and AP1A cooperated in basolateral protein exit from the TGN. Endosome ablation experiments showed that AP1A knockdown directed basolateral proteins into a transendosomal route to the PM. Our experiments suggest that AP1A controls a direct biosynthetic route from TGN to the basolateral PM while AP1B controls transendosomal and recycling routes. Selected reading 1. S. Deborde et al., Nature 452, 719 (2008). 2. H. Folsch, H. Ohno, J. S. Bonifacino, I. Mellman, Cell 99, 189 (1999).

2451/B159

Clathrin Regulates Basolateral Protein Sorting at the Golgi Complex and Recycling Endosomes.

S. Deborde, D. Gravotta, E. Perret, A. Deora, S. Salvarezza, R. Schreiner, E. Rodriguez-Boulan; Weill Medical College Cornell Univ, New York, NY

Clathrin-coated vesicles are vehicles for intracellular trafficking in all nucleated cells, from yeast to humans. Many studies have demonstrated their essential roles in nutrient uptake and cellular signalling processes at the plasma membrane. By contrast, very few of their intracellular roles are known; their best characterized one is the transport of hydrolases from the Golgi complex to the lysosome. Recently we have demonstrated a requirement for clathrin in the transport of basolateral plasma membrane proteins in MDCK cells (Deborde et al. Nature, 452:719-723, 2008). Clathrin knock-down by siRNA depolarized 5 out of 6 basolateral proteins studied, covering a broad range of basolateral signals but did not affect the polarity of apical proteins. Loss of basolateral protein polarity was accounted for by interference with their biosynthetic delivery and recycling. Quantitative live imaging showed that chronic clathrin knock-down and acute block of clathrin function by cross-linking inhibited the exit of basolateral proteins from the Golgi complex and promoted their incorporation into apical carrier vesicles. Using MDCK cells expressing Ds-Red-clathrin, we have also shown in two color movies that a basolateral proteins (VSVG-GFP) leaves the Golgi complex in clathrin coated vesicles. Our results demonstrate a broad requirement for clathrin in the biosynthetic sorting of basolateral plasma membrane proteins in epithelial cells.

2452/B160

Delayed Assembly of AP1 Complexes in Fisher Rat Thyroid Epithelial Cells.

C. Torrealba[2,3], J. Cancino[1,3,2], A. Gonzalez[1,2,3]; [1]Inmunología Clínica y Reumatología, Facultad de Medicina, P. Universidad Católica de Chile, Santiago, Chile, [2]Centro Fondap de Regulación Celular y Patología, Facultad de Ciencias Biológicas, P. Universidad Católica de Chile, Santiago, Chile, [3]MIFAB, Santiago, Chile

The AP1B member of the family of tetrameric adaptor protein (AP) complexes has been involved in basolateral sorting in polarized epithelial cells. AP1 is composed of four subunits: two large (beta and gamma), one medium (mu A and B) and one small subunit (sigma). The two known AP1 isoforms, AP1A and AP1B, differ only in their medium subunit mu1A and mu1B, sharing 80% homology. AP1B is specifically expressed in certain epithelial cells and plays a basolateral sorting role in post-TGN recycling endosomes. We previously described antibodies against the N-terminal region of mu1B that recognize the endogenous protein by immunofluorescence and blocked AP1B-dependent basolateral trafficking in Fisher rat thyroid (FRT) but not in Madin-Darby canine kidney (MDCK) cells (Cancino et al MBC-2007). However, dog and rat mu1B share almost identical N-terminal sequences and structural data reveals inaccessibility of this region to antibodies. Here, we performed comparative pulse-chase and immunoprecipitation experiments of AP1A and AP1B complexes using anti-gamma subunit antibodies. FRT cells showed a several hour delay in the incorporation of the beta-mu heterodimers into these complexes, with respect to MDCK cells. This delayed assembly of AP1 complexes could leave N-terminal mu1B accessible to interaction with antibodies, resulting in function blocking effects exclusively in FRT cells (Financed in part by FONDECYT grant# 1050715, FONDAP grant# 13980001 and Millenium Project, Ministerio de Planificación).

2453/B161

The C-terminus of µ1B Is Required for Recruitment of AP-1B onto Recycling Endosomes.

I. C. Fields, S. King, E. Shteyn, R. S. Kang, H. Folsch; Biochemistry, Molecular Biology & Cell Biology, Northwestern University, Evanston, IL

Polarized epithelial cells separate their plasma membrane into distinct apical and basolateral domains. In order to maintain this polarity, epithelial cells must continuously sort their transmembrane proteins to either apical or basolateral domains during biosynthetic and endocytic trafficking. The epithelial cell-specific clathrin adaptor complex AP-1B is central for sorting of transmembrane proteins with tyrosine-based sorting signals from perinuclear common recycling endosomes to the basolateral plasma membrane domain. Here we asked what features of AP-1B determine its localization to recycling endosomes as opposed to the TGN where its close cousin AP-1A localizes. These different localizations are remarkable given that AP-1A and AP-1B differ only in one out of four subunits (Fölsch et al., 1999). Furthermore, the medium subunits mu1A or mu1B are 79% identical on the amino acid level and they have overlapping cargo specificity (Ohno et al., 1999). We identified a conserved amino acid patch within the C-terminus of mu1B that is crucial for recruitment of AP-1B onto recycling endosomes and its function in basolateral sorting. Furthermore, we identified a signature lipid for recycling endosomes of polarized, AP-1B-positive epithelial cells. Manipulating the lipid composition of recycling endosomes with pharmacological inhibitors or specific lipid phosphatases leads to a displacement of AP-1B from recycling endosomes and apical missorting of AP-1B-dependent cargo. These data explain on a molecular level why AP-1A and AP-1B do not co-localize and highlight the importance of intact recycling endosomes for AP-1B-mediated sorting to the basolateral membrane.

2454/B162

MAL2 Selectively Regulates Polymeric IgA Receptor Delivery from the Golgi to the Plasma Membrane in Hepatic Cells.

J. G. In, P. Tuma; Biology, the Catholic University of America, Washington, DC

Unlike simple epithelial cells that directly target proteins from the TGN to the apical PM, hepatocytes use an indirect pathway: proteins are first delivered to the basolateral domain, then selectively internalized and transcytosed to the apical surface. MAL2 has been identified as a regulator of indirect apical delivery and is proposed to function between the basolateral early endosome and sub-apical compartment. However, overexpression of polymeric immunoglobulin-A receptor (pIgA-R) led to the dramatic redistribution of MAL2 into all transcytotic intermediates and the Golgi, with near perfect co-localization between MAL2 and the receptor. Although overexpressed dipeptidyl peptidase IV (DPP IV) and hemagglutinin (HA) distributed to the same compartments as pIgA-R, MAL2 distributions did not change indicating the effect is selective for pIgA-R. Treatment with cycloheximide led to the eventual loss of pIgA-R and MAL2 intracellular staining first in the Golgi then in the SAC suggesting they were delivered together to the apical PM and that MAL2 may be mediating that process. This was confirmed in studies in Clone 9 cells (that lack endogenous MAL2 expression). When expressed alone, pIgA-R staining was mainly restricted to the Golgi whereas when coexpressed with MAL2, the receptor was detected at the PM and in intracellular puncta that also contained MAL2. Surface delivery of newly synthesized pIgA-R and DPPIV was measured, and only pIgA-R delivery was greatly enhanced (> 5-fold) by MAL2 co-expression. Thus, we have determined that in addition to its proposed role in regulation of transcytosis from the early endosome to the SAC, MAL2 also regulates pIgA-R delivery from the Golgi to the PM. To test this, we performed coimmunoprecipitations and found that less than 1% of pIgA-R coimmunoprecipitated with MAL2. However, no DPPIV was recovered in the MAL2 immunoprecipitates, suggesting the pIgA-R-MAL2 interactions are specific, but likely weak, transient, or indirect. We favor the latter possibility and are currently identifying MAL2 binding partners to further examine its interactions with pIgA-R and other apical proteins.

2455/B163

The Role of Rip11/FIP5 and Rab11 Complex in Regulating Protein Transport to Apical Plasma Membrane in Polarized Epithelial Cells.

C. Willenborg[1], R. Cameron[1], H. Matern[2], C. Wu[3], J. Burden[4], R. Prekeris[1]; [1]Cell and Developmental Biology, University of Colorado Denver, Aurora, CO, [2]Exelixis, South San Francisco, CA, [3]Pharmacology, University of Colorado Denver, Aurora, CO, [4]MRC Cell Biology Unit, University College London, London, United Kingdom

Epithelial cells are structurally and functionally polarized to transport specific molecules while maintaining trans-epithelial barrier. This transport is achieved by partitioning the plasma membrane into two distinct domains: apical and basolateral. Apical transport is fundamental

to the function of all epithelial cells and malfunctions in a number of diseases. Significant progress in understanding the mechanisms regulating basolateral sorting of proteins has been made, however, the machinery involved in apical protein sorting and targeting remains largely unknown. Rab11 GTPases are members of the small monomeric GTPase super-family that have been implicated in regulating apical endocytic transport. In the last few years several Rab11-binding proteins have been identified, including the Rab11 family interacting proteins, also known as FIPs. In this study we show that Rip11/FIP5 is highly enriched at the apical pole of polarized MDCK cells as well as in the rat kidney tubules. Furthermore, we use MDCK cell line, stably-expressing Rip11/FIP5 shRNA, to demonstrate that Rip11/FIP5 plays a key role in protein transport to the apical plasma membrane, as well as, in primary cilia formation and maintenance. Furthermore, we used electron microscopy analysis of Rip11/FIP5 dominant negative mutant expressing cells to show that Rip11/FIP5 is required for the protein exit from tubulo-vesicular recycling endosomes. Work from several laboratories, including our own, have shown that FIPs act as scaffolding factors allowing the assembly of specific sorting/transport complexes required for the endocytic protein traffic. Thus, used proteomics to identify Sorting Nexin 18 (SNX18) as a Rip11/FIP5-binding protein. We have mapped Rip11/FIP5 and SNX18 binding motifs and shown that Rip11/FIP5 may regulate SNX18 binding to lipids in vitro and the targeting of SNX18 to the endosomes in vivo. Finally, we created a MDCK cell line that is stably expressing SNX18 shRNA to test the role of SNX18 in epithelial cell polarization and cilia formation. Based on these data, we propose that the binding of Rab11 and Rip11/FIP5 protein complex to SNX18 mediates the budding/sorting of apical endocytic proteins carriers in epithelial cells.

2456/B164

Synaptogyrin2: a Putative Regulator of Membrane Recycling.

J. Williams[1,2], L. A. Lapierre[1,3], J. Goldenring[1,2,3]; [1]Surgery, Vanderbilt University Medical Center, Nashville, TN, [2]Cell & Developmental Biology, Vanderbilt University Medical Center, Nashville, TN, [3]Nashville VA Medical Center, Nashville, TN

The delivery of proteins to the appropriate membrane domain is essential for proper cell function. There are numerous proteins including small GTPases and their effectors that direct/regulate these processes. We previously performed a proteomic analysis on gastric tubulovesicles, a rich source of Rab11a containing recycling vesicles, one of the proteins identified in this study was Synaptogyrin2 (SYNGR2). SYNGR2 is a ubiquitously expressed member of the Synaptogyrin family that has been reported to play a role in membrane recycling. We have generated an antibody against the carboxy-terminus of SYNGR2 that by western blot analysis and immuno-fluorescence is specific for SYNGR2 and does not cross react with any other family members. Immunofluorescent staining of Sloan Kettering-Colonic (SkCo-15) cells revealed two pools of SYNGR2 positive vesicles, (1) pericentrally localized - that partially co-localized with endogenous Rab11a and (2) a population juxtaposed to the lateral plasma membrane. Overexpression of Rab11-Family Interacting Protein 2(FIP2) mutant constructs, known to collapse the apical recycling system, caused some endogenous SYNGR2 to get sequestered into the collapsed apical recycling compartment. Similarly, the overexpression of the myosin Vb tail also caused partial pull in of the endogenous SYNGR2 towards the collapsed Rab11a recycling system. Together, these findings support the involvement of a pool of Synaptogyrin2 containing vesicles within the Rab11 apical membrane recycling pathway.

2457/B165

Endosomes Sort Apical Cargo into Distinct Transport Carriers.

C. I. Cramm-Behrens, R. Jacob; Cytobiology, Philipps University Marburg, Marburg, Germany

Epithelial polarity is based on intracellular sorting machinery that maintains the asymmetric distribution of lipids and proteins to the cell surface. Dependent on their lipid raft affinity newly synthesized apical polypeptides are segregated into distinct vesicle populations subsequent to the passage through the Golgi apparatus. Using a combined fluorescence microscopic and biochemical approach, we found that lipid raft-associated sucrase isomaltase (SI) as well as non-raft-associated lactase-phlorizin hydrolase (LPH) traverse endosomal compartments before entering the apical membrane. Fluorescent fusion proteins of both hydrolases were co-stained with Rab4-, Rab8- and Rab11-positive endosomes in polarized MDCK and non-polarized COS-1 cells. Immunoisolation of post Golgi vesicles subsequent to different times of TGN-release revealed that LPH and SI navigate in chronological order through Rab4-, Rab8- and Rab11-positive endosomes. Thereafter, the two hydrolases are segregated into distinct vesicle populations. In addition, apical membrane traffic could be significantly blocked by RNAi-mediated depletion of Rab8. These results suggest that lipid raft dependent and independent pathways of apical cargo in polarized cells partition in an endosomal compartment.

2458/B166

Quantitative Imaging of the FcRn-Dependent Transcytotic Pathway Using High-Resolution Fluorescence Confocal Microscopy.

G. V. Jerdeva, D. Tesar, P. Bjorkman; Biology, California Institute of Technology, Pasadena, CA

The neonatal Fc receptor (FcRn) transports maternal (IgG) across intestinal or placental epithelial barriers in the apical to basolateral direction to provide immunity to newborn or fetal mammals and serves as a protection receptor for IgG. We investigate FcRn-mediated transport of IgG or Fc and compare it to the transport of dimeric and polymeric IgA (dIgA/pIgA) mediated by the well studied polymeric immunoglobulin receptor (pIgR). **Objective:** We wish to identify at what stages of endocytosis / transcytosis the trafficking routes of Fc and pIgA intermix, and to learn the identities of endosomal compartments involved in receptor trafficking. **Methods:** We are using Madin-Darby Canine Kidney (MDCK) cells expressing either one or both receptors (MDCK-FcRn, MDCK-FcRn-pIgR). The transfected cells specifically transcytose IgG or Fc and pIgA across polarized cell monolayers when applied apically and basolaterally, respectively. A spinning disk Confocal Imaging System (UltraVIEW ERS) with a sensitive EMCCD Camera allows acquisition of high resolution 3D imaging volumes of cells after internalization of fluorescently-labeled ligands co-stained with endogenous intracellular markers with minimal bleaching of fluorescent probes. **Results:** Quantitative colocalization analysis of 3D volumes of florescent ligand exposed cells revealed significant colocalization of apically internalized Fc and basolaterally internalized pIgA after 20 min of chase but not after 5 min, as reflected in calculations of the amount of colocalizations between two channels (13.95% + 0.99% at 20 min chase; 1.13% + 0.3% at 5 min chase) and Pearson's correlation coefficients (P) (P=0.38 + 0.01 at 20 min chase ; P=0.05 + 0.007 at 5 min chase). A significant subpopulation of ligand-positive endosomes at the 20 min chase were also positive for the early endosomal marker EEA1 (10.9% + 1.30% for Fc; 9.66% + 1.67 for

pIgA). P values for Fc /EEA1 and pIgA / EEA1 in regions of interest were 0.33 + 0.2 and 0.30 + 0.03, respectively, indicating significant spatial overlap in internalized ligands and the marker. **Conclusions:** FcRn and pIgR trafficking routs do intermix in later time chase intervals, in part in sub-apical EEA1-positive endosomes.

2459/B167

Clathrin Regulates Membrane Localization of Gβ and Asymmetric Spindle Positioning in C. Elegans Embryos.

K. Thyagarajan, K. Afshar, P. Gönczy; School of Life Sciences, EPFL SV ISREC, Epalinges, Switzerland

Asymmetric cell division is a conserved mechanism used to generate diversity during development. The first division of the *C. elegans* embryo is unequal as a result of the posterior displacement of the spindle, which gives rise to daughters of different sizes. Asymmetric spindle positioning is due to unbalanced net pulling forces acting on the two spindle poles, and depends on the presence of GOA-1 and GPA-16, the Gα subunits of the heterotrimeric G protein complex, and their interplay with their positive (GoLoco protein GPR-1/2, coiled-coil protein LIN-5 etc.) and negative (Gβ protein GPB-1, Gγ protein GPC-2 etc.) regulators. In the wild-type, these proteins are enriched at the cell cortex, where the pulling forces are thought to be exerted on astral microtubules. The mechanisms by which these proteins are localized to the cell cortex are poorly understood. We found that compromising the function of clathrin using RNAi leads to increased pulling forces. Interestingly, in these embryos, GPB-1 is no longer present on the cell membrane, but is found instead in RAB-7 positive intracellular endosomal compartments. We found that the depletion of other proteins involved in intracellular trafficking e.g. RAB-5, DYN-1 etc. also lead to a mislocalization of GPB-1 and an alteration of pulling forces. We conclude that GPB-1 traffics through the endosomal system in wild-type embryos, and propose that clathrin and other trafficking components play an important role in modulating asymmetric spindle positioning in *C. elegans* embryos.

2460/B168

Genome-Wide Rnai Screen for Regulators of Membrane Trafficking and Polarity Maintenance in the C.Elegans Intestinal Epithelium.

J. F. Winter[1], S. Hoepfner[1], B. Farnung[1], C. Bradshaw[1], K. Korn[2], M. Volkmer[3], B. Habermann[3], M. Zerial[1]; [1]Zerial laboratory, Max-Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, [2]Technology Developmental Studio (TDS), MPI-CBG, Dresden, Germany, [3]Bioinformatics Group, MPI-CBG, Dresden, Germany

Epithelial cells define the interface between the internal and external milieu of virtually all multicellular organisms. Their structural and functional polarity comprises two biochemically distinct membrane surfaces, an apicobasally polarized cytoskeleton and a sophisticated membrane trafficking system. The 'apical recycling endosome' (ARE) sustains the distinctness of the apical and basolateral plasma membrane and of membrane organelles by recycling and sorting endocytosed membrane cargo. Its apically enriched steady state position is typical for the polarized organization of the epithelial membrane trafficking system. Yet very little is known about the molecular mechanisms that regulate the structural and functional properties of this important organelle. In order to elucidate the molecules coupling the organization of membrane traffic to epithelial polarity maintenance, we conducted a genome-wide RNAi screen in an *In Vivo* system, the *C.elegans* intestine. Utilizing two fluorescently tagged markers, we identified candidates that maintain the trafficking of an apical membrane protein and the spatial distribution and morphology of the ARE. As a reference, effects on autofluorescent lysosome-related organelles were also analyzed. The distribution of organelles and cargo were monitored by automated acquisition of 2.2 million fluorescent images that were processed by a custom-built image analysis software. Out of 16,757 genes analyzed, about 400 mainly conserved candidates exhibited an altered marker distribution. The most enriched group in the hit set represented membrane trafficking genes, underscoring the validity of our screening approach. After hierarchical clustering of phenotype profiles assigned to all candidates, 70 selected candidates were analyzed in morphological secondary RNAi assays. With this systematic survey, we identified numerous established traffic regulators, such as the exocyst complex, and novel candidates with known and unknown molecular functions. These candidates are required for specific membrane trafficking routes, for the steady state position of distinct membrane compartments and for the maintenance of epithelial polarity.

2461/B169

Type II Phosphatidylinositol 4-Kinase Is Required for Glue Granule Biogenesis in Drosophila.

J. Burgess[1,2], M. Jauregui[1,2], H. Kramer[3], J. Brill[1,2]; [1]Molecular Genetics, University of Toronto, Toronto, ON, Canada, [2]Developmental and Stem Cell Biology, Hospital for Sick Children, Toronto, ON, Canada, [3]Center for Basic Neuroscience, UT Southwestern Medical Center, Dallas, TX

The lipid phosphatidylinositol 4-phosphate (PI4P) is synthesized by PI 4-kinases (PI4Ks). PI4P plays a crucial role in post-Golgi trafficking by recruiting several clathrin adaptor proteins, including the AP-1 complex, epsinR and GGA. We report the first analysis of a type II PI4K in the multicellular organism *Drosophila melanogaster* and identify a novel role for PI4KII in granule biogenesis. Flies bearing deletions that remove the sole *Drosophila PI4KII* (*dPI4KII*) gene are viable, male sterile and exhibit defects in eye pigmentation and salivary gland granule biogenesis. These phenotypes can be rescued by a *dPI4KII* rescue transgene. To investigate a potential role of dPI4KII in post-Golgi trafficking, we focused on the specialized secretory cells of the larval salivary gland. Highly glycosylated 'glue proteins' are synthesized specifically in the salivary gland beginning at the mid-third instar larval stage. By late third instar, mature glue granules fill the entire cytoplasm of salivary cells, and subsequently fuse with the apical membrane in response to a pulse of the steroid hormone 20-hydroxyecdysone. *dPI4KII* mutants accumulate strikingly small glue granules, as revealed by the granule specific marker SNAP-24. Semi-thin sections of *dPI4KII* mutant salivary glands confirm this observation and also reveal the presence of large vacuoles. Furthermore, late endosomal compartments appear enlarged as detected by Rab7 immunostaining. Mosaic analysis of single salivary gland cells mutant for *dPI4KII* in an otherwise wild-type background reveal that dPI4KII is required cell autonomously for glue granule biogenesis. dPI4KII localizes to vesicular structures, which likely represent intermediate transport vesicles. To identify potential adaptor proteins that might function downstream of PI4P for glue granule biogenesis, we knocked down epsinR and AP-1γ using RNAi in the salivary gland. Both epsinR and AP-1γ knockdown results in stunted salivary glands with small granules. We propose that glue granule biogenesis represents a novel system in which to investigate post-Golgi trafficking events and implicate dPI4KII as a key regulator in this process.

2462/B170

Roles of Bud7p in Unconventional Trafficking System of Cortical Polarity Landmarks, Bud8p and Bud9p, and Role of Ste20p (PAK kinase) in Cell-Polarity Establishment in Saccharomyces Cerevisiae.

K. Nakashima, S. Tuo, J. Pringle; Department of Genetics, Stanford University School of Medicine, Stanford, CA

Asymmetric periphery organization is the foundation of cell polarity in many different cell types such as epithelial cell, stem cell, zygote. In *S. cerevisiae*, the asymmetric localization of integral-membrane proteins such as Bud8p and Bud9p respectively localize to distal pole and proximal pole referred to the birth scar (division scar separated from mother cell). They are homologous to each other and both play a role for the landmark of polarity-establishment machinery in diploid cell, which produce buds exclusively from the polar zone of cell (bipolar budding). We found Bud8p and Bud9p are indefinitely stabile, suggesting their trafficking to special membrane domain. The localization and function of Bud8p and Bud9p are required Bud7p, one of homologues of Chs6p, the trafficking regulator of chitin synthase III. But they are delivered to normal sites after pulse expression. Both Bud8p and Bud9p show N-link glycosylation in 20-40 min after expression, suggesting that they stay in cytoplasm for a while before ER translocation. During this period, Bud8p and Bud9p are stably maintained by Bud7p and delivered to the polarity site dependent on F-actin filament and Rax1p (landmark component), respectively. To investigate the significance of this cytoplasm period, we construct Bud8p and Bud9p mutant, which translocate to ER simultaneously with translation by tagging with the signal peptide. They are defective function and localization, suggesting long cytoplasmic period is important. Interestingly, the mutants exhibit peripheral scattered-localization pattern, but they are not co-localized with Sur7p, the component of one of dotty localized lipid rafts. This suggests that Bud8p and Bud9p are occupying yet unknown raft space. The peripheral localization of these mutants is depending on Bud7p, so Bud7p plays a role for their association with lipid raft and secretion to plasma membrane. All suggest Bud8p and Bud9p trafficking to stable membrane domain is by unconventional system associated with Bud7p. Ste20p is one of the PAK kinase involved in distal pole landmark in vegetative cell. Any known substrates are not related to the phenotype. So we are searching the substrate to link Ste20p to the phenotype.

2463/B171

Rsr1, a Ras-like GTP-ase, Influences Vesicle Trafficking in Candida Albicans Hyphae.

R. Pulver[1], C. A. Gale[1,2]; [1]Genetics, Cell Biology, and Development, University of Minnesota, Minneapolis, MN, [2]Pediatrics, University of Minnesota, Minneapolis, MN

The highly polarized morphology of hyphae contributes to virulence of the opportunistic yeast pathogen C. albicans. For example, deletion of the C. albicans Ras-like GTP-ase, Rsr1, causes defects in hyphal tip polarity, tissue damage, and invasion. A model of hyphal growth in Aspergillus nidulans, a filamentous fungus, proposes that endocytic cycling at the hyphal tip is important for polarized morphogenesis. C. albicans strains lacking Rsr1 show abnormal localization and morphology of cortical actin patches. In addition, a vesicle-rich structure normally located at hyphal tips, the Spitzenkörper, is destabilized. These phenotypes suggest possible endosome trafficking defects. Differential localizations of Rsr1's GTP-ase activating protein (GAP), Bud2, and its guanine nucleotide exchange factor (GEF), Bud5, suggest that Rsr1 activity is controlled at the hyphal tip. Thus, we hypothesized that Rsr1 activity influences endocytic cycling at hyphal tips. To test this hypothesis we used the lipophilic dye FM4-64 in conjunction with fluorescence recovery after photobleaching (FRAP) to compare vesicle dynamics at the hyphal tips of rsr1 null, bud2 null, and control strains. We found that rsr1 null strains have reduced rates of fluorescence recovery to hyphal tips as compared to controls. In contrast, bud2 null strains showed no defect in the rate of fluorescence recovery, but did show a reduction in the total fluorescence recovered to the region of interest. In preliminary experiments, neither rsr1 nor bud2 null strains showed any difference in fluorescence recovery at regions of the hypha located away from the tip. This supports the idea that Rsr1 activity is restricted to the hyphal tip. In summary, these results suggest that Rsr1 is important for vesicle trafficking in C. albicans hyphae. In addition, although deletion of Bud2 does not affect the rate of vesicle trafficking, it appears to influence vesicle dynamics within the region of the Spitzenkörper. We speculate that the bud2 null phenotype results from an enrichment of GTP-bound Rsr1 at hyphal tips.

Endocytosis II (2464 – 2479)

2464/B172

Quantitative Assay for Notch Internalization and Trafficking in Mammalian Cells.

E. Sorensen, S. Conner; Genetic, Cell biology and Development, University of Minnesota, Minneapolis, MN

The Notch signaling pathway is important for cell differentiation and proliferation. Notch signaling activity must be tightly regulated because both overactive signaling or its absence has been linked to cancer in humans. Published observations demonstrate that the clathrin-mediated endocytic pathway is critical in modulating both the activation and down-regulation of Notch signaling events. Although endocytosis is clearly important, the regulatory steps involved in controlling the Notch signaling pathway are not well resolved. To gain insight into how Notch signaling activity is regulated through the endocytic pathway, we developed a quantitative Notch internalization assay for both the inactive and activated forms of the receptor using mammalian tissue culture cells. Consistent with published reports, our preliminary studies add support for the importance of the clathrin-mediated endocytic pathway in receptor activation. Analyzing the kinetics of internalization for activated Notch, we find that >40% of cell surface receptor is rapidly internalized at rates comparable to that observed for nutrient receptors, like the transferrin receptor. Moreover, we find that Notch internalization rates are significantly reduced following overexpression of dominant-negative constructs that are known to disrupt the clathrin-dependent endocytic pathway (i.e. K44A dynamin1 and T102A Numb). We are currently using this newly developed assay to identify regulatory factors that are essential for coordinating Notch internalization and intracellular sorting decisions within the endosome.

2465/B173

E-Cadherin Endocytosis Is Regulated by Cdc42-Par6-aPKC via WASp and Arp2/3 in Drosophila Epithelial Cells.

A. Leibfried[1], R. Fricke[2], M. J. Morgan[1,3], S. Bogdan[2], Y. Bellaiche[1]; [1]UMR144 CNRS, Institut Curie, Paris, France, [2]Institute for Neurobiology, University of Muenster, Muenster, Germany, [3]Department of Human Genetics, University of Leuven, Leuven, Belgium
Epithelial adherens junction (AJ) stability and plasticity relies on E-Cadherin (E-Cad) exocytosis and endocytosis, but the interplay of possible regulators to orchestrate these events remains to be understood. Here we have analyzed the role of Cdc42 in the regulation of AJ maintenance in *Drosophila* epithelial cells. We show that loss of function of Cdc42 and the polarity proteins Par6 and aPKC is accompanied by the accumulation of E-Cad in apical punctate structures and the disruption of AJs. These punctate structures are endocytic vesicles as demonstrated by Cy3-labelled E-Cad antibody uptake. Furthermore they derive from large and malformed vesicles that emanate from the AJs, a phenotype that is also observed upon blocking vesicle scission in *dynamin* mutant cells. The Cdc42 effector WASp and its target Arp2/3 seem to regulate E-Cad endocytosis and E-Cad homeostasis at the AJs downstream of Cdc42/Par6/aPKC. Our results establish that Cdc42 functions with Par6 and aPKC to regulate vesicle scission and therefore E-Cad endocytosis via WASp and Arp2/3. Thus the interplay of polarity proteins and actin regulators such as the Arp2/3 complex is necessary for the homeostasis of endocytic trafficking of E-Cad in epithelial cells.

2467/B175

Endocytic Trafficking of GABAB Receptors in Central Neurons.

K. J. Vargas[1,2], M. Terunuma[3], J. A. Tello[1], S. J. Moss[3,4], A. Couve[1,5]; [1]Physiology and Biophysics, Universidad de Chile, Santiago, Chile, [2]Universidad Austral de Chile, Valdivia, Chile, [3]Department of Neuroscience, Tufts University School of Medicine, Boston, MA, [4]Department of Pharmacology, University College London, London, United Kingdom, [5]Center for Integrated Neurosciences, Santiago, Chile
The efficacy of synaptic transmission depends on the availability of ionotropic and metabotropic neurotransmitter receptors at the plasma membrane, but the contribution of the endocytic and recycling pathways in the regulation of GABAB receptors remains controversial. To understand the mechanisms that regulate the abundance of GABAB receptors we have studied their turnover combining surface biotin labeling and a microscopic immunoendocytosis assay in hippocampal and cortical neurons. We report that internalization of GABAB receptors is agonist-independent. We also demonstrate that receptors endocytose in the cell body and dendrites but not in axons. Additionally, we show that GABAB receptors endocytose as heterodimers via clathrin and dynamin-1 dependent mechanisms, and that they recycle to the plasma membrane after endocytosis. More importantly, we show that glutamate decreases the levels of cell surface receptors in a manner dependent on an intact proteasome pathway. These observations indicate that glutamate and not GABA controls the abundance of surface GABAB receptors in central neurons, consistent with their enrichment at glutamatergic synapses. *Supported by CONICYT D-21050545 and 24071048 (KJV); FONDECYT 1071001 and Iniciativa Científica Milenio ICM-P04-068-F (AC); The American Heart Association (MT).*

2469/B177

Resolution of Signaling Endosomes Containing Different Receptors.

M. Grimes; Division of Biological Sciences, University of Montana, Missoula, MT
Gretchen McCaffrey*§, Jonathan Welker*§ Jessica Scott*, Louise van der Salm‡, and Mark L. Grimes*§ *Division of Biological Sciences and §Center for Structural and Functional Neuroscience, University of Montana, Missoula, Montana; ‡Institute of Molecular Biosciences, Massey University, Palmerston North, New Zealand Cell surface receptors continue to signal after endocytosis. Signaling from endosomes may even activate downstream signaling pathways different from those activated by receptors at the plasma membrane. It is not known, however, whether signaling endosomes are specialized organelles different from the canonical endosomal traffic for recycling or degradation, or if different receptors internalize to the same or different signaling endosomes. Signaling endosomes containing TrkA, activated by nerve growth factor (NGF) are thought to play a pivotal role governing the survival and differentiation of neurons. We hypothesize that TrkA signaling endosomes employ special sorting mechanisms that are distinct from canonical endocytosis pathways. If specialized signaling endosomes exist, they may have different physical properties due to different components. We devised an organelle fractionation method based on mass and density that resolves endosomes from pheochromocytoma and neuroblastoma cell lines. Endosomes containing Rab5, Rab4, and Rab7 were resolved from one another by this method, indicating that different populations of endosomes have different mass and density. Surprisingly, we found that the physical properties of endosomes containing the receptors TrkA, p75NTR, and PAC1 were resolved from one another. The data suggest that signaling receptors employ distinct sorting mechanisms during endocytosis. Signaling endosomes may activate effectors in different ways. The time course after NGF stimulation of APPL1 and GIPC association with endosomes was different; APPL1 associated and dissociated more rapidly than GIPC. We conclude that endosomes containing different signaling receptors can be distinguished by physical characteristics and by the time course of effector activation.

2470/B178

The Hydrolysis of PI(4,5)P2 by the Synaptojanin-Endophilin Partnership Is Modulated by Membrane Curvature.

B. Chang-Ileto, S. Voronov, G. Di Paolo; Department of Pathology and Cell Biology, Columbia University, New York, NY
Studies have implicated a fundamental role of $PI(4,5)P_2$ in the synaptic vesicle (SV) cycle. Based on growing evidence suggesting that $PI(4,5)P_2$ hydrolysis is critical for the endocytic retrieval of SVs, we have hypothesized that the $PI(4,5)P_2$ hydrolysis machinery, which includes the major synaptic phosphoinositide phosphatase synaptojanin 1, may utilize membrane curvature generators/sensors, such as BAR proteins, to remove this lipid preferentially from curved membranes rather than flat ones. This hypothetical mechanism would allow for the effective elimination of $PI(4,5)P_2$ from endocytic membranes during the invagination process and, conversely, for the maintenance of adequate $PI(4,5)P_2$ levels at the plasma membrane, which is required for coat assembly as well as multiple other functions. To test this hypothesis, we employed cell-free assays relying on the incubation of brain cytosol with liposomes of varying diameters/curvatures. We found that $PI(4,5)P_2$ hydrolysis occurs more efficiently on highly curved membranes (50 nm in diameter) than on relatively flat membranes (800 nm in diameter). This phenomenon can be abolished by depletion of synaptojanin 1-interacting BAR proteins (such as endophilin) or synaptojanin 1 from brain cytosol. In addition, synaptojanin 1 is recruited preferentially to small liposomes in the presence of endophilin, suggesting that the abilty of these two molecules to physically interact is potentiated by highly-curved membranes. Altogether, our data suggest that the ability to sense membrane curvature by BAR proteins may be a mechanism to spatially restrict the hydrolysis of $PI(4,5)P_2$ by

synaptojanin to areas of endocytosis where the budding and fission process produces membranes of high curvature. This study expands on previous work from others showing that membrane curvature plays an important role in the regulation of proteins, such as small GTPase of the Arf family, which mediate key aspects of membrane budding reactions.

2471/B179

PtdIns(3,4,5)P3-Induced Receptor Tyrosine Kinase Endocytosis Requires Polarity Complex Proteins.

V. Laketa[1, 2], S. Zarbakhsh[1], R. Pepperkok[2], C. Schultz[1]; [1]Gene Expression, EMBL, Heidelberg, Germany, [2]Cell Biology and Biophysics, EMBL, Heidelberg, Germany

Receptor tyrosine kinases (RTK) stimulation by various growth factors leads to the activation of type I phosphatidylinositol 3-kinase (PI3K) and thus to the transient formation of 3,4,5-trisphosphate [PtdIns(3,4,5)P3] and phosphatidylinositol 3,4-bisphosphate [PtdIns(3,4)P2]. Down-regulation of RTKs is mainly achieved by endocytosis of the receptor and subsequent lysosomal degradation. However, very little is known about the role of PtdIns(3,4)P2 and PtdIns(3,4,5)P3 in receptor internalization. To address this, we used membrane-permeant derivatives of PtdIns(3,4)P2 and PtdIns(3,4,5)P3. In contrast to traditional methods for manipulating intracellular phosphoinositide levels such as down-regulation or over-expression of respective kinases or phosphatases, this new technique allowed us to alter intracellular phosphoinositide levels within minutes, resulting in a more physiological mimicking of growth factor signalling. Membrane-permeant PtdIns(3,4)P2 and PtdIns(3,4,5)P3 derivatives activated signaling events downstream of phosphatidylinositol 3-kinase (PI3K) within minutes of addition, including mitogen-activated protein kinase (MAPK), protein kinase B, p70S6K, and protein kinase C, while similar PtdIns(4,5)P2 derivative was inactive. Both, PtdIns(3,4)P2 and PtdIns(3,4,5)P3, but not the 4,5- or 3,5-bisphosphates, were sufficient to induce clathrin-mediated endocytosis of epidermal growth factor receptor (EGFR) in the absence of ligand. Other transmembrane proteins such as G-protein coupled receptors, ion channels or GPI anchored proteins were not affected. Surprising, downregulation of all proteins containing a membrane binding domain by a high-throughput siRNA screen revealed a major role of the polarity complex and actin binding proteins in PtdIns(3,4,5)P3-induced EGFR endocytosis. The combined results demonstrate that PtdIns(3,4,5)P3 constitutes a sufficient signal to induce EGFR endocytosis and therefore could function as a second messenger in RTK endocytosis. The specificity of the endocytosis for RTKs suggests a sorting mechanism that most likely involves PtdIns(3,4)P2 and/or PtdIns(3,4,5)P3 and the respective phosphoinositide binding proteins.

2472/B180

The SPG20 Spastic Paraplegia Protein Spartin Is Ubiquitinated by AIP4.

Z. A. Loring, A. Marchese, J. C. Bakowska; Pharmacology, Loyola University Chicago, Maywood, IL

Hereditary spastic paraplegia type 20 (SPG20) is caused by mutation of the spartin gene. We have recently found that spartin is an adaptor endocytic protein involved in the internalization and trafficking of the epidermal growth factor receptor. We have also shown that spartin is constitutively mono-ubiquitinated. Notably, spartin contains a PPXY motif, which is known to interact with WW domains of E3 ubiquitin ligases belonging to the Nedd4 family. The objectives of the current studies were to identify the E3 ligase responsible for spartin's ubiquitination and to determine the role that ubiquitnated spartin plays in the trafficking of receptor tyrosine kinases. Using co-immunoprecipitation, we learned that spartin associates with atrophin-1 interacting protein 4 (AIP4) but not Nedd4_2. Immunofluorescence microscopy studies demonstrated that over-expressed spartin co-localizes with AIP4 in punctuated structures in the cell. We also found that expression of the dominant negative construct, AIP4 C830A (mutated in the enzymatic catalytic activity domain) results in a marked decrease in spartin ubiquitination. In order to further characterize the interaction between spartin and AIP4, we performed in vitro "pull-down" assays using various constructs of GST-AIP4 and over-expressed spartin. These experiments demonstrated that spartin binds preferentially to the WWII domain and to a lesser degree to the WWI domain of AIP4. Moreover, mutation of spartin in its PPAY domain revealed that spartin Y174A does not interact with GST-AIP4 nor does it co-immunoprecipitate with AIP4. Taken together, our results strongly suggest that spartin is ubiquitinated by the E3 ligase AIP4 and that the interaction between these proteins occurs primarily between the PPAY domain of spartin and the WWII domain of AIP4. We are currently investigating the functional effect of spartin Y174A in the sorting of specific receptor tyrosine kinases.

2473/B181

Regulation of EGFR Cell Surface Dynamics by TI-VAMP Dependent Transport of CD82.

L. Danglot[1,2,3], M. Chaineau[1,2,3], M. Dahan[7], M. Gendron[3,4], N. Boggetto[3,4], F. Perez[5,6], T. Galli[1,2,3]; [1]Cell Biology, INSERM Avenir Team: Membrane Traffic in Neuronal & Epithelial Morphogenesis, Paris, France, [2]Cell Biology, CNRS, UMR7592, Institut Jacques Monod, Univ Paris 6 & Paris 7, Paris, France, [3]Univ Pierre et Marie Curie-Paris6 and Univ Paris Diderot-Paris7, Paris, France, [4]CNRS, UMR7592, Institut Jacques Monod, Plateforme de Cytométrie en flux, Univ Paris 6 & Paris 7, Paris, France, [5]CNRS, UMR144, Paris, France, [6]Institut Curie, Paris, France, [7]Laboratoire Kastler Brossel, CNRS, Physics Department, Ecole Normale Supérieure, 24, Rue Lhomond, Paris, France

A major function of the endocytic pathway is to sort internalized proteins for degradation and/or recycling. The vesicular SNARE TI-VAMP/VAMP7 was previously involved in fusion processes required for EGF to reach the lysosomal degradative compartment, but little is known about its precise function in EGF-EGFR trafficking. Here we show that the silencing of TI-VAMP specifically increased EGFR endocytosis without affecting its amount at the cell surface or its degradation. Single particle tracking showed that this effect was associated with decreased cell surface diffusion of EGFR in TI-VAMP silenced cells. The vesicular SNARE TI-VAMP/VAMP7 was previously involved in exocytosis mediating growth of the plasma membrane during neurite outgrowth, phagocytosis and mitosis. We thus presumed that TI-VAMP could regulate cell surface dynamics of EGFR through the transport of regulators at the plasma membrane. Four-transmembrane-domain proteins of the tetraspanin superfamily are the organizers of specific microdomains at the membrane. Here, we found that TI-VAMP regulates the transport of the tetraspanin CD82 at the cell surface. Indeed, depletion of TI-VAMP decreased the expression of CD82 at the plasma membrane. Furthermore, ultra-fast confocal videomicroscopy indicates that RFP-TI-VAMP and YFP-CD82 are transported in the same vesicles. Silencing of CD82 increased EGF and EGFR endocytosis and decreased EGFR diffusion at the cell surface.

Altogether, these results demonstrate that TI-VAMP-dependent transport regulates EGFR endocytosis and dynamics at the cell surface by controlling the secretion of CD82.

2474/B182

The Hominoid-Specific Oncogene TBC1D3 Activates Ras and Modulates Growth Factor Receptor Signaling and Trafficking.

P. Srikanth[1], M. Wainszelbaum[1], C. Kong[1], D. Hodzic[1], X. Su[2], P. Stahl[1]; [1]Cell Biology & Physiology, Washington University Medical School, St. Louis, MO, [2]Internal Medicine, Washington University School of Medicine, St. Louis, MO

Hominoid- and human specific genes may have evolved to modulate signaling pathways of a higher order of complexity. TBC1D3 is a hominoid-specific oncogene encoded by a cluster of 8 paralogs on Chromosome 17. Initial work indicates that TBC1D3 is widely expressed in human tissues (Hodzic et. al., 2006). In this study, we show that TBC1D3 expression has a powerful effect on cell proliferation that is further enhanced by epidermal growth factor (EGF) in both human and mouse cell lines. Moreover, Erk and PKB/Akt activation responses were increased after stimulation with EGF and other growth factors (i.e. PDGF and IGF-1) in cells expressing TBC1D3; meanwhile RNA silencing suppressed that activation. Light microscopy and western blot experiments demonstrate that increased signaling in response to EGF is coupled with a significant delay in EGFR trafficking and degradation, which significantly extends the life span of EGFR. Moreover, using confocal microscopy we show that TBC1D3 significantly co-localizes with EGFR at the plasma membrane. Using the Ras Binding Domain of Raf1 to monitor GTP-Ras we show that TBC1D3 expression enhances Ras activation in quiescent cells, which is further increased by EGF or serum treatment. Immunoprecipitation in cells transfected with TBC1D3 and Grb2 showed these proteins interact. In vitro transcription/translation and pull-down experiments using GST-coupled proteins confirmed a direct interaction between TBC1D3 and the SH2 domain of Grb2. We hypothesize that TBC1D3 acts as a potent oncogenic trigger enhancing Ras activation in response to serum and growth factor receptors through its interaction with Grb2. Altered growth factor receptor trafficking and GTP-Ras turnover may be sites where recently evolved genes such as TBC1D3 selectively modulate signaling in hominoids and humans.

2475/B183

Mechanism Underlies Internalization Event and Signaling Compartmentalization of Human GM-CSF Receptors.

P. Chen[1,2], J. Yen[2]; [1]Graduate Institute of Life Sciences, National Defense Medical Center, Taipei, Taiwan, [2]Institute of Biomedical Sciences,Academia Sinica, Taipei, Taiwan

Granulocyte-macrophage colony stimulating factor (GM-CSF) receptor consists of two subunits, the α chain and the common beta chain (βc). The βc chain is shared by IL-3, IL-5, and GM-CSF receptors, and the α chain is specific to its ligands. Upon GM-CSF binding, GM-CSF receptors transmit signals to modulate cellular proliferation, differentiation, and mobilization. Our laboratory previously found that βc chain recruited an apoptogenic protein, designated as common beta chain associating protein (CBAP), during GM-CSF deprivation and participated in apoptosis modulation. The mechanism by which βc chain distinguishes divergent signaling processes in hemopoietic cells is not well studied. Recently, many studies suggest that internalized receptors upon ligands binding not only are destined for degradation but also have active regulative roles in receptors signaling. Here we show that the βc chain was constantly internalized independent of its ligand. Deletion of the C-terminus retained the βc chain as well as the α chain on plasma membrane. It indicates the C-terminus of βc chain has an internalization signal for its endocytosis. By serial and internal deletion analyses, we identified that the box 1 and its flanking region are responsible for βc chain internalization. Since that this region is also necessary for JAK kinase association and that JAKs association was reported to target receptors to surface, we explored whether JAK kinases are also required for receptor internalization. We found JAKs are dispensable for βc internalization by siRNA knockdown of endogenous JAKs, but are indispensable for βc signaling. It implies that βc is internalized through proteins other than JAKs while the internalization motif is overlapped with the JAK binding region. The mechanism by which internalized GM-CSF receptors α and β chains used to modulate the survival and death signals will be explored.

2476/B184

Tyr-Phosphorylation Signals Translocate RIN3, the Small Gtpase Rab5-GEF, to Early Endocytic Vesicles.

H. Kajiho, M. Yoshikawa, K. Sakurai, T. Minoda, S. Nakagawa, K. Kontani, T. Katada; Dept. of Physiol. Chem.,Grad. School of Pharm. Sci., The University of Tokyo, Tokyo, Japan

The small GTPase Rab5 plays a key role in early endocytic pathway, and its activation requires guanine-nucleotide exchange factors (GEFs). Rab5-GEFs share a conserved VPS9 domain for the GEF action, and RIN3 containing additional domains, such as Src-homology 2, RIN-family homology (RH) and Ras-association (RA), was identified as a new Rab5-GEF. However, precise functions of the additional domains and the activation mechanism of RIN3 remain unknown. Here this study's object is to elucidate the roles of these domains according to the stimulation involved with tyrosine-phosphorylation signals. We found tyrosine-phosphorylation signals are involved in the Rab5-GEF activation. Treatment of HeLa cells with pervanadate translocates RIN3 from cytoplasm to the Rab5-positive vesicles. This RIN3 translocation was applied to various mutants lacking each domain of RIN3. Our present results suggest that a Ras GTPase(s) activated by tyrosine-phosphorylation signals interacts with the inhibitory RA domain, resulting in an active conformation of RIN3 as a Rab5-GEF and that RIN-unique RH domain constitutes a Rab5-binding region for the progress of GEF action.

2477/B185

Cdc42 and Dynamin-2 Are Involved in Shiga Toxin 1 Macropinocytosis by Gb3-Negative Human Intestinal Epithelial Cells.

I. Malyukova, O. Kovbasnjuk; Medicine, Johns Hopkins University, Baltimore, MD

The mechanism of Shiga toxin (Stx) endocytosis by human intestinal epithelial cells (IEC) which normaly do not express Stx receptor a globotriaosylceramide Gb3 is poorly understood. We have demonstrated that macropinocytosis might be potentially responsible for Stx uptake by IEC. Macropinocytosis is known to be regulated by small GTPases, including cell division cycle 42 (Cdc42), while the role of the GTP-binding protein dynamin is controversial. We investigated the role of Cdc42 and dynamin-2 in macropinocytosis of Stx1 in human IEC. Human colonic epithelial T84 cell monolayers were treated with N-ethylmalemide (NEM) to stimulate the macropinocytosis and the uptake of Stx1 B-subunit (Stx1B) directly conjugated to Alexa 680 fluorescent dye was measured in total cell lysates by fluorescent plate reader.

The involvement of Cdc42 in NEM-stimulated Stx1B uptake was tested by inhibition its activity by pirl-l, a specific blocker of nucleotide exchange on Cdc42 or by shRNA technique. The role of dynamin-2 in NEM-stimulated Stx1B uptake was tested using dynasore a specific inhibitor of dynamin GTPase activity, or by shRNA. To deplete Cdc42 or dynamin T84 cells were transduced with lentivirus particles bearing shRNA to Cdc42 or to dynamin-2. Pirl-1 inhibited Stx1 B endocytosis in a dose-dependent manner. Decrease in Cdc42 expression by shRNA prevented NEM-stimulated Stx1B uptake. Decrease in dynamin activity by dynasore prevented NEM-stimulated endocytosis of Stx1 B in T84 cells. Depletion of dynamin by shRNA decreased NEM-stimulated uptake of Stx1 B up to the control level. We concluded that NEM-stimulated macropinocytosis of Stx1B in Gb3-negative human intestinal epithelial T84 cells is Cdc42 and dynamin-2 dependent and pharmacological or molecular inhibition of Cdc42 or dynamin-2 functioning significantly downregulates Stx1B macropinocytosis.

2478/B186

Identification of Two Novel Intracellular Partners of the Fractalkine Receptor.

A. Chamberland[1], A. Pelletier[1], C. Laprise[1,2], V. Legendre-Guillemin[1]; [1]Sciences fondamentales, Université du Québec à CHicoutimi, Chicoutimi, QC, Canada, [2]Centre de médecine génique communautaire du CSSSC, Chicoutimi, QC, Canada

CX3CR1 is a G-coupled protein receptors (GPCRs). Upon activation, many GPCRs are internalized through clathrin-mediated endocytosis and are subsequently sorted for recycling or lysosomal degradation. However, the regulation of the cell surface expression of the CX3CR1 is not yet characterized. In order to characterize the dynamics of this receptor, we first generated four GST-fusion proteins emcompassing the four intracellular domains of CX3CR1 and we performed pull-down experiments from different tissue extracts in order to identify intracellular binding partners of CX3CR1. Two novel intracellular binding partners from liver extract and specific to the second cytoplasmic region of CX3CR1 (126-142) have been identified by mass spectrometry. These proteins were GAPDH and Hsc70, these interactions have been confirmed by western blot. We also demonstrated that the binding was direct for Hsc70 using purified Hsc70. To further characterize these interactions, we created deletion and point mutations of the 126-142 region. The 126-136 was targeted as essential for both interactions. We also identified that the D126N and the I131A point mutations abolished the binding to CX3CR1 for both GAPDH and Hsc70. The next steps will be to verify how these binding partners are implicated in the function of the receptor in particular in its endocytosis, its signaling and its chemotaxis properties.

2479/B187

Mechanism and Role of CD82 Trafficking.

M. M. Richardson[1], B. Zhou[1], X. Zhang[1,2]; [1]Vascular Biology Center for Excellence and Department of Medicine, University of Tennessee Health Science Center, Memphis, TN, [2]Department of Molecular Sciences, University of Tennessee Health Science Center, Memphis, TN

CD82, a tetraspanin, is a tumor metastasis suppressor. Tetraspanins are transmembrane spanning proteins that play critical roles in diverse biological processes. CD82 is ubiquitously expressed on several types of normal tissues but lost in a majority of metastatic tumor cell lines. It inhibits tumor metastasis without affecting primary tumor growth. Cancer patients whose tumors contain CD82 have a minimal amount of metastasis to occur. We know that CD82 functions as a tumor suppressor but the mechanism by which this occurs is largely unknown. CD82 can be found on the plasma membrane as well as in intracellular compartments. The mechanism by which CD82 is able to internalize remains unknown. CD82 contains a YXXΦ internalization and intracellular trafficking motif in its c-terminal domain. Previous reports suggest that mutating the YXXΦ motif of a tetraspanin will disrupt internalization. We have established a transfectant that contains a mutation in the YXXΦ motif of CD82. Therefore, this research focuses on the role of CD82's YXXΦ motif in intracellular trafficking as well as in migration of metastatic tumor cells. Results from the internalization assays reveal that the YXXΦ motif is not important for the internalization of CD82, nor does it have an effect on the steady state distribution of CD82. Results from migration assays reveal that the mutation of the YXXΦ motif significantly enhances the suppression of cell migration on laminin 1. We also found that the YXXΦ motif does indeed play a role in the recycling pathway of CD82. From the results obtain from this study, we conclude that the YXXΦ motif plays a role in intracellular trafficking of CD82. Further studies are ongoing to determine the mechanism by which this occurs.

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2480/B189

Real-time Imaging Reveals That MDCK Tubulogenesis Is Occurred by Cell Movement, Rearrangement, and Evagination.

M. Kim[1], A. J. Ewald[1], J. Engelberg[2], Z. Werb[1], C. A. Hunt[2], K. Mostov[1]; [1]Anatomy, UCSF, San Francisco, CA, [2]Bioengineering and Therapeutic Sciences, UCSF, San Francisco, CA

Hepatocyte growth factor (HGF), a large multifunctional polypeptide growth and motility factor, is known to play important roles during embryonic development and tissue repair. In a three-dimensional type I collagen model, HGF induces Madin-Darby canine kidney (MDCK) epithelial cysts to form long, branching tubules (tubulogenesis). We have previously proposed that tubulogenesis can be divided into two stages, dedifferentiation and re-differentiation. However, cellular events that occur during HGF-mediated remodeling remain unclear. To determine how epithelial cells organize themselves into tubes, we visualized either nuclei or/and plasma membrane using GFP-histone or/and GFP-c-terminal region of K-ras or GFP-actin and examined the mechanism of tubulogenesis by real-time microscopy. With HGF treatment, polarized MDCK cells protrude basally from the cyst (extension), which is HGF dose-dependent. However, most cell protrusions produced by HGF are retracted or detached. Thus, increased sprouting of cell protrusion does not directly result in the increased tubule formation. As cells acquire the motility concomitant with proliferation and filopodial extensions, the structures transforms into a long multicellular chains and cords. After ECM triggers repolarization, cells in a cord develop luminal surfaces (vacuole like) and create lumens at the apical region. To form a complete lumen in a tubular structure, which is connected with the cyst lumen, "evagination" at the cyst wall takes place and luminal space is expanded until it merges. We propose a model for HGF induced tubulogenesis driven by the combined mechanism of cell movement, rearrangement, and evagination.

2481/B190

UNC-53 Functions Independently of VAB-8 to Control Cell Migration.

N. Marcus[1,2], E. Stringham[1,2]; [1]Biology, Trinity Western University, Langley, BC, Canada, [2]Molecular Biology & Biochemistry, Simon Fraser University, Burnaby, BC, Canada

In C. elegans, unc-53/NAV-2 (uncoordinated-53/Neuron Navigator-2) is required for cell migration and neuron outgrowth. UNC-53 binds F-actin and SEM-5/GRB2 implicating UNC-53 in both signal transduction and cytoskeletal dynamics (Stringham, et al., 2002). Recently, we have shown that UNC-53, through its CH domain, binds ABI-1 and predict it acts as a scaffold to link ABI-1 to the ARP2/3 complex to regulate actin cytoskeleton remodeling (Schmidt et al., submitted). The gene vab-8 (variable abnormal-8) encodes a kinesin-like motor domain required for many posteriorly directed migrations, and is implicated in localizing receptors such as SAX-3/ROBO to the cell surface (Wightman et al., 1996; Watari-Goshima et al., 2007). unc-53 and vab-8 mutants display defects within some of the same longitudinally migrating cells including the excretory canals and mechanosensory neurons (Stringham et al., 2002; Wightman et al., 1996). Misexpression of VAB-8 in the ALM mechanosensory neuron results in the normally anteriorly directed axon to be rerouted posteriorly, an effect that is enhanced in unc-53 mutants (Wolf et al., 1998). These results suggest that either UNC-53 negatively regulates VAB-8 or that UNC-53 and VAB-8 operate in separate pathways to control migration. unc-53(n166); vab-8(e1017) mutants display an enhanced canal extension defect compared to either single null allele, suggesting the genes act in separate pathways with each exerting their influence independently on the migration of the excretory canals. A candidate RNAi screen and construction of double mutants revealed genes implicated as interactors of unc-53 and/or vab-8, including unc-6/netrin, unc-71/ADAM, unc-73/TRIO, and sax-3/ROBO. While sax-3 (ky123) mutants are wild type, in a sensitized heterozygous unc-53(n166) background, the excretory canals showed disruption as severe as in unc-53(n166) homozygotes. This may reveal a compensatory role for sax-3, or alternatively SAX-3/ROBO and UNC-53 may physically interact.

2482/B191

Molecular Control of Cell Migratory Capacity in C. Elegans Q Neuroblasts.

G. Ou, R. Vale; Dept. of Cellular and Molecular Pharmacology, HHMI / University of California - San Francisco, San Francisco, CA

Cell migratory capacity needs to be precisely regulated in metazoan organisms; either too little or too much cell migration can cause developmental disorders or cancer. Cell migration distance and speed can be measured to characterize a cell's migratory capacity. We have developed live cell imaging methodologies to visualize C. elegans Q neuroblasts development, and we found that each of Q descents has distinct migratory capacities. The QL neuroblast at the Left lateral divides into QL.a (anterior) and QL.p (posterior) progeny, and QL.a and its progeny QL.ap migrate towards the posterior but neither QL.p nor QL.pa moves. QR neuroblast at the Right lateral behaves differently; all QR descents can move anteriorly to the head region, but QR.a and QR.ap migrate much faster and further than their sisters QR.p and QR.pa respectively. Thus, the distinct migration patterns of Q cells provide excellent models to understand the molecular control for cell migratory capacity at single cell resolution. Previous genetic screens for abnormal Q neuroblast development identified dozens of mutants defective in its migration, and we are currently performing time-lapse imaging of these mutants to understand the nature of the migratory defects. We also are imaging the levels of critical molecules for cell migration (e.g. Rho family GTPase and adhesion proteins) by GFP tagging. With a combination of imaging approaches in developing C. elegans, we hope to address how very distinct cell migratory capacity are conferred to the different daughter cells during the asymmetric cell divisions of the Q neuroblasts.

2483/B192

Increased Cell Motility in Upside-Down Cells Is Src Family Kinase Dependent.

T. Thomas, Y. Chiu, K. Fujiwara; Aab Cardiovascular Research Institute, University of Rochester, Rochester, NY

Cells respond to a variety of stimuli both chemical and mechanical, and various techniques have been used in the study of cellular response to mechanical forces such as stretch, fluid shear stress, hypo/hyper osmotic shock, and the employment of magnetic beads and atomic force microscopy. Gravity is another form of mechanical force that cells are exposed to. Simulated microgravity experiments have indicated that cells, especially their cytoskeletal organization, are affected by microgravity. These results suggest that cells are capable of responding to gravity. We wondered if the direction of the gravitational force would affect cells. Cultured bovine aortic endothelial cells in Sykes-Moore chambers were left rightside-up or turned upside-down for several hours, exposing them to an inversion of gravitational direction. Time-lapse recording of cells revealed that the upside-down cells moved significantly faster (24 ± 10%) than the rightside-up counter parts. In addition, the upside-down cells developed many lamellipodia which was observed by anti-Arp3 staining. We also found that tyrosine phosphorylation of FAK increased in upside-down cells. Because Src family kinases are involved in FAK phosphorylation, we treated cells with PP2 and studied their response to reversed gravitational direction. PP2 inhibited cell motility of rightside-up cells by 13 ± 3%, but it inhibited the motility of the upside-down cells more significantly such that their motility (20 ± 5%) became statistically indistinguishable from the extent of cell migration of the rightside-up cells in PP2. This result indicates that Src family kinase activity is responsible for the increased motility of upside-down cells. The result further suggests that Src family kinase is involved in cells response to gravity.

2484/B193

High Glucose-associated Oxidative Stress Impairs Cell Migration.

M. Lamers[1,2], M. Vicente-Manzanares[2], A. Horwitz[2], M. F. Santos[1]; [1]Cell and Developmental Biology, University of São Paulo, São Paulo, Brazil, [2]Cell Biology, University of Virginia, Charlottesville, VA

Diabetes is characterized by chronic hyperglycemia, which promotes metabolic changes and oxidative stress. Deficient wound healing is a common event associated to diabetes. However, the reason underlying this effect is not known, and the possible direct effect of high glucose on cell migration remains unclear. Our objective was to investigate if high glucose concentrations exert a direct inhibitory effect on cell migration. CHO.K1 cells and NIH-3T3 fibroblasts were cultured in 5mM D-glucose (low glucose, LG), 25 mM D-glucose (high glucose, HG) or 25mM L-glucose (osmotic control, OC) media; then analyzed for migration-related parameters. The results show that HG inhibited cell migration compared to LG or OC, and also induced an increase of the frequency of non-productive protrusions. Such effect was associated with a marked decrease in protrusion persistence and stability as well as a significant reduction in adhesion maturation, which

suggests that non-productive protrusiveness is due to deficient adhesion. HG also impaired cell polarity. All these phenotypic changes were associated to a modest increase of Rac1 activity. Most of these effects of HG were partially or completely reverted by treatment with antioxidants such as N-acetyl-cysteine. In conclusion, high glucose concentration impairs cell migration due to an increase in oxidative stress that causes polarity loss, deficient adhesion and non-productive protrusiveness. The mechanism is not yet clear, but it involves increased activation of the small GTPase Rac1. Financial support: NIH, CAPES, FAPESP.

2485/B194

CD36 Modulates Macrophage Spreading and Migration in Response to Oxidized LDL.

Y. M. Park[1,2], M. Febbraio[1], R. L. Silverstein[1]; [1]Department of Cell Biology, Lerner Research Institute, the Cleveland Clinic Foundation, Cleveland, OH, [2]Graduate Program in Cell Biology, Case Western Reserve University, Cleveland, OH

CD36 is a class B scavenger receptor that is expressed on a variety of cells including macrophages, adipocytes, platelets and microvascular endothelial cells. It has been implicated in many biological processes including atherosclerosis, angiogenesis, innate immunity, and thrombosis. Previous work has shown that CD36 binds to various ligands including oxidized LDL (oxLDL), bacterial cell wall components, apoptotic cells and thrombospondin-1, and transmits intracellular signals. We hypothesized that macrophage CD36 may modulate cytoskeletal function and thus affect macrophage adhesion and migration. RESULTS: We found using both in vivo and in vitro assays that oxLDL but not native LDL inhibited migration of wild type murine macrophages but not CD36 null cells. OxLDL also induced rapid spreading and actin polymerization in CD36 expressing, but not null macrophages. The mechanism of these effects was shown to be dependent on oxLDL mediated CD36 signaling, which resulted in sustained activation of focal adhesion kinase (FAK) and inactivation of src homology 2-containing phosphotyrosine phosphatase (SHP-2). The latter was due to NADPH oxidase mediated generation of reactive oxygen species (ROS) with resulting oxidative inactivation of critical cysteine residues in the active site of SHP-2. Immuno-precipitation with anti-CD36 antibody revealed that CD36, FAK and SHP-2 are physically associated. Macrophage migration in the presence of oxLDL was restored by treating the cells with antioxidants or NADPH oxidase inhibitors which also restored dynamic activation of FAK. CONCLUSIONS: CD36 signaling in response to oxLDL alters cytoskeletal dynamics to enhance spreading and inhibit migration. This suggests that CD36 may be an important modulator of macrophage migration under pro-inflammatory conditions and oxidant stress. This may induce trapping of macrophages in inflammatory sites such as within the neointima of atherosclerotic plaque.

2486/B195

FGF and VEGF Signaling Control Cell Movements during Streak Formation in the Chick Embryo.

M. Chuai, C. Weijer; Cell & Developmental Biology, College of Life Sciences, University of Dundee, Dundee, United Kingdom

We study the initiation and extension of the primitive streak during gastrulation in the chick embryo. Using timelapse microscopy we have characterized the detailed movements of randomly GFP labeled cells in the epiblast during streak formation. These experiments show that the cells undergo large scale co-ordinated cell movements, which are organized in two counter-rotating cell flows, which merge at the site of streak formation. These movements transport the forming mesoderm into the midline of the embryo. Major questions are which signals coordinate these movements and by which mechanisms the cells in the one cell layer thick epithelial sheet of the epiblast move? It has been proposed that streak formation is driven by a cell-cell intercalation mechanism of cells at the base of the forming streak, a process possibly controlled by the Wnt Planar polarity signaling pathway. Our observations do not support a major role for cell intercalation in streak formation and blocking of the Planar polarity signaling pathway at several different points does not interfere with streak formation. We therefore investigate the alternative hypothesis that streak formation is driven by a combination of chemo-attractive and repulsive cell movements. Experiments directed towards the identification of potential signaling molecules show that FGF's and VEGF's are important players, since upregulation of these factors block the cell movements associated with streak formation. Investigation of the mechanism of cell movement, through detailed high magnification observations of cellular dynamics using actin-GFP and VASP-GFP, show that epiblast cells extend numerous highly dynamic basal protrusions especially on the basal lamina between the epiblast and the hypoblast. This strongly suggests that the epiblast cells move actively and get traction from this matrix. At present we investigate whether and how FGF and VEGF signaling affect these cell behaviors.

2487/B196

Melanoma Migratory Behavior Is Modulated by ET-1 and the Rho Family Small GTPases.

K. M. Teal, R. W. Baer; Physiology, A. T. Still University of Health Sciences, Kirksville, MO

Endothelin 1 (ET-1) has been identified as a key molecule in melanoma epithelial-mesenchymal transition (EMT) and tumor progression. One hallmark of the EMT is an increased ability to migrate and invade surrounding tissues. Signaling molecules of the Rho family small GTPases such as RhoA and Rac1 are key regulators of actin-dependent cell functions including cell morphology, adhesion, and migration. We hypothesized that ET-1 would increase melanoma cell migration through RhoA- and Rac1-dependent mechanisms. To measure migration we used a wound closure assay of the B16 F10 mouse melanoma cell line. B16 F10 melanoma cells were grown to confluence in 24-well plates with RPMI-1640 media and 10% fetal bovine serum. Migratory distance was assessed from images taken using the 2.5x objective on a Zeiss Axiovert 200 inverted microscope. The average migratory distance of a single wound edge was calculated as: (Change wound area) / (2*wound length). Treatment of B16 F10 melanoma with ET-1 (10 nM) at time of wound initiation resulted in increased wound closure that depended on the activation of the Endothelin B receptor (ETBR). Inhibition of RhoA signaling with the ROCK inhibitor Y-27632 (30 µM, 24 h) decreased ET-1-induced wound closure. In addition, inhibition of ROCK changed the mode of migration from collective migration with intact cell-cell adhesions to a mesenchymal-like migration pattern with few cell-cell adhesions. Inhibition of Rac1 with NSC23766 (50 µM, 24 h) dramatically increased wound closure. Subsequent stimulation with ET-1 slowed closure but not back to untreated control levels. These data show that ET-1 influences B16 F10 melanoma migration in Rho family GTPase-dependent manner. The RhoA effector, ROCK, appears to be essential for ET-1- induced increases in melanoma migration; whereas, Rac signaling appears to have a more complex interaction with ET-1 signaling.

2488/B197

A Simple Model of Moving Fish Epidermal Keratocyte Shapes.

K. Lee[1], A. Mogilner[2,1]; [1]Neurobiology, Physiology and Behavior, University of California, Davis, Davis, CA, [2]Mathematics, University of California, Davis, Davis, CA

Fish epidermal keratocytes, like other motile eukaryotic cells, crawl by using forces generated by F-actin polymerization and Actin-Myosin contraction. Unlike other cells, moving keratocytes polarize into and maintain fan-like, static shapes, instead of irregular shapes. This highly symmetric shape implies that only a few parameters are needed to characterize the shape. In a recent article [Nature 453, 475 (2008)], it is found that one parameter, the ratio of membrane tension to concentration of diffusing G-actin at the center of lamellipodium, controls the shape of the moving front of keratocytes. In this work, we extend the analysis to develop a model describing the shape at the backend of moving keratocytes. We show, using symmetry and continuity arguments, that the shape at the backend is also necessarily determined by a handful of parameters, which are related to the strength of contraction.

2489/B198

Isolation and Culture of Motile C. Elegans Sex Myoblast Cells for High-Resolution Microscopy.

S. Zhang, J. Kuhn; Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA

Light microscopy of isolated cells moving on two-dimensional substrates has driven our understanding of the actin motility machinery. Although the amoeba Dictyostelium discoideum has been the primary model system for microscopy, its genome is highly redundant, complicating the dissection of the actin-based motility machinery. The nematode Caenorhabditis elegans has a more concise genome and offers a wealth of genetic tools, but single-cell observations have been limited to non-motile embryonic primary cells. Somatic cells from larval and adult worms, including the genetically well-characterized motile sex myoblast (SM) cells, have thus far been difficult to culture owing to the collagenase-resistant cuticle. We have successfully isolated somatic cells from L2 worms using a combination of collagenase treatment and RNA interference (RNAi) targeting of DUOX-1 that catalyzes collagen crosslinking. Cells from small-scale preparations adhere to poly-L-lysine coated coverslips, can be cultured for at least 3 days, and have well defined nuclei and actin cytoskeletons. Because the efficiency of this RNAi method was limited, we developed an alternative large-scale method to break 90% of the L2 worms by extensive vortexing with 3 mm glass beads. Labeling of myoblast (M) lineage with hlh8::GFP-CAAX showed that M lineage cells, which included SM, remain intact and survive culture conditions. Taken together, our attempt to culture SM cells is promising. A C. elegans model system of cell motility will bring the wealth of available RNAi and mutant libraries, targeted gene knockouts, in vivo imaging, biochemical tractability, and rapidity and ease of worm culture to bear on the dissection of the cell motility machinery in multicellular organisms.

2490/B199

Identification of Amino Acids in the Tre1 G Protein-coupled Receptor Important for Cell Migration and Programmed Cell Death.

M. M. Pruitt, A. R. Kamps, C. R. Coffman; Genetics, Development and Cell Biology, Iowa State University, Ames, IA

Cell migration is essential for proper development and homeostasis in many animals. Defects in this process are implicated in many diseased states, such as cancer metastasis. Germ cell development in Drosophila melanogaster is an excellent system to study this process. In fact, Drosophila primordial germ cells (PGCs) have similar characteristics to metastatic cells; both need to initiate migration, travel through different layers of tissue and evade cell death mechanisms. The PGCs in Drosophila are formed at the posterior pole and are passively swept into the posterior midgut primordium during gastrulation. The PGCs then actively migrate out of the midgut, into the lateral mesoderm and to the presumptive gonads. Germ cells that do not migrate properly are eliminated by programmed cell death (PCD). In a severe loss of function allele of tre1, tre1sctt, germ cell migration and PCD are disrupted. The germ cells in this mutant scatter across the posterior half of the embryo as opposed to forming two gonads. Few, if any, germ cells make it to the gonads and the ectopic germ cells are not eliminated by PCD. Tre1 is a G-protein coupled receptor (GPCR) that functions in the germ cells. GPCRs are a family of receptors that mediate signaling initiated by a large number of extracellular cues. Despite the growing body of evidence that GPCRs are involved in cell migration and cell death, signaling downstream of Tre1 is uncharacterized. The molecular lesion in tre1sctt has been identified as an eight amino acid deletion. Structure-function analyses were performed in vivo and resulted in the identification of the key amino acids within this eight amino acid deletion of Tre1 that are important for normal germ cell migration and PCD in Drosophila. These results will help elucidate the signaling networks downstream of Tre1 and have the potential to identify molecules that directly interact with this region of the Tre1 GPCR to mediate signaling.

2491/B200

Kif5C Localization in Migrating Neurons.

E. C. Kao[1], J. C. Shieh[1,2], B. T. Schaar[3], S. K. McConnell[1]; [1]Department of Biology, Stanford University, Stanford, CA, [2]Neurosciences Program, Stanford University, Stanford, CA, [3]Department of Neurosurgery, Stanford University, Stanford, CA

An early step in the formation of functional neural circuits is the migration of neurons to appropriate positions in the developing brain. Neuronal migration is a saltatory process during which a leading process extends in the direction of movement, followed by the translocation of the cell body into this process. Leading processes often branch as neurons explore the environment through which they are moving; however, these branches must be consolidated into a single leading process for cell body movement to occur efficiently. The neuron's choice to retain a single branch may be analogous to the process of neuronal polarization in which a single neurite is specified as the axon. Previous studies have demonstrated that the constitutively active motor domain of the kinesin, Kif5C, can function as the earliest known marker of axonal identity, since it accumulates and persists in the neurite that becomes the axon (Jacobson et al., 2006, Neuron 49:797). Here, we investigate whether Kif5C acts similarly in migrating neurons as they select a single leading process. We transfected a construct containing a truncated Kif5C sequence fused to YFP ($Kif5C^{560}$-YFP) into migrating neurons to observe Kif5C localization within these cells. In fixed neurons that displayed a migratory morphology, $Kif5C^{560}$-YFP accumulated at the tip of the leading process. During time-lapse imaging, $Kif5C^{560}$-YFP was seen to localize to the tip of the leading process as the process extended and retracted. These results suggest that the

localization of Kif5C may predict which branch is retained as the leading process, hence predicting the direction of movement in migrating neurons.

2492/B201

ENaC Mediates Directional Keratinocyte Migration in an Electric Field.

H. Yang[1], R. Charles[2], E. Hummler[2], A. M. Woolhead[3], D. L. Baines[3], R. Isseroff[1]; [1]Dermatology, University of California, Davis, Davis, CA, [2]Pharmacologie & Toxicologie, Université de Lausanne, Lausanne, Switzerland, [3]Ion Channels and Cell Signaling Research Centre, St. George's University of London, London, United Kingdom

Ion channels and exchangers have been proposed to regulate cell migration by conducting ion flow at lamellipodia and anchoring actin filaments. Here we report a novel function of the epithelial sodium channel, ENaC, in galvanotaxis, the directional cell migration in an electric field and an important mechanism to facilitate re-epithelialization during wound healing. ENaC regulates sodium transport in kidney and lung, and it is also found in epidermis and may mediate epidermal differentiation. By time-lapse imaging, we monitored neonatal human keratinocytes (NHK) galvanotaxis toward cathode in a 100mV/mm electric field. Our results demonstrated that the keratinocyte directionality, but not the velocity, in galvanotaxis decreased by 30% with 20μM phenamil, an ENaC inhibitor, and by 80% in keratinocytes from αENaC knockout mice and in αENaC siRNA-knockdown NHK. Additionally, a human lung epithelial cell line H441 expressing a low level of ENaC does not show directional migration in the electric field; however, when GFP-αENaC is over-expressed in H441, the cells showed a significant increase of directionality during galvanotaxis. Therefore, ENaC plays an important role of mediating the galvanotaxis of human and mouse keratinocytes. In contrast, inhibiting the sodium-proton exchanger 1 by cariporide did not block NHK galvanotaxis, suggesting that it is not solely sodium flux that governs galvanotaxis of NHK. To further study if ENaC-mediated galvanotaxis is involved in re-epithelialization, we examined the healing of normal human skin wound in organ culture. In the presence of phenamil, the re-epithelialization was blocked by 70% compared to control. To elucidate a possible mechanism of ENaC-mediated galvanotaxis, we examined ENaC localization during galvanotaxis. ENaC or GFP-ENaC did not polarize at the leading edge of NHK or H441 in the early stage of galvanotaxis. When we treated NHK with the inhibitory peptide LPHPLQRL derived from αENaC, galvanotaxis was significantly inhibited by 30% vs the control, which suggests that the protease-cleaved, active αENaC is required for galvanotaxis. Therefore, ENaC might mediate galvanotaxis by local activation at the cathodal side of NHK but not by its polarized localization.

2494/B203

Role of Small GTPases Rap and Ral in LPA Stimulated Breast Cancer Cell Migration and Invasion.

M. Alemayehu, T. Li, M. Bhattacharya, C. Pape, J. Law; Physiology and Pharmacology, University of Western Ontario, London, ON, Canada

Lysophosphatidic acid (LPA) is a simple phospholipid found in blood that signals through its specific G protein-coupled receptors (LPA1-3) to mediate a variety of cellular processes including cell migration and invasion, required for cancer metastasis. Recently, LPA has been shown to stimulate metastasis of breast cancer cells to bone, although the specific mechanisms by which this occurs are largely unknown. The small G protein Ral has been shown to stimulate proliferation of breast cancer cells. The activity of Ral can be regulated by Ral Guanine-Dissociation Stimulator (RalGDS) as well as the small G protein Rap1. In order to investigate the possible role LPA and its downstream components play in tumorigensis, we compared the mRNA expression of LPA receptors, Ral, RalGDS, and Rap1A in the normal mammary epithelial cell line MCF-10A and the invasive breast cancer cell line MDA-MB-231 as well as the melanoma cell line MDA-MB-435. Our results showed that both cancer cell lines have increased mRNA expression of LPA1 receptor, and Rap1A compared to MCF-10A cells. Using Transwell-chamber migration/invasion assays as well as three-dimensional morphogenesis assays, we show that MDA-MB-231 cells migrate and invade in response to LPA stimulation via a Ral-and Rap-dependent pathways. In summary our results show for the first time that Ral and Rap GTPAses are involved in mediating LPA-stimulated breast cancer migration and invasion.

2495/B204

Release of CD44 from Lipid Raft Microdomains May Govern Ezrin Interactions during Stimulated Breast Cancer Cell Migration.

S. Donatello, A. Hopkins; Surgery, Royal College of Surgeons in Ireland, Dublin, Ireland

CD44 is a transmembrane receptor for the extracellular matrix component hyaluronic acid. A key intracellular binding partner of CD44 is ezrin, which acts as a linker between the membrane and the actin cytoskeleton during cell migration. CD44 has been described to localize in cholesterol-enriched plasma membrane microdomains called lipid rafts. Although altered expression or membrane localization of CD44 has been implicated in many carcinomas, little is known about the role of lipid rafts in regulating CD44/ezrin interactions during cancer cell motility. Thus the objective of our study was to determine if co-localization of CD44 and ezrin in lipid rafts is important for breast cancer cell migration. Confluent MDA-MB-231 invasive breast cancer cells were scratch-wounded to stimulate migration, or left unwounded as non-migrating controls. Lipid rafts were isolated by sucrose density gradient fractionation using Triton X-100 or sodium carbonate. CD44/ezrin immunoprecipitations were performed in raft versus non-raft fractions. Confocal immunofluorescence microscopy was also used to assess CD44 or ezrin localization to lipid rafts. Our results show that CD44 and ezrin localize in different cellular compartments in MDA-MB-231 cells. CD44 was enriched in lipid raft fractions, while ezrin was recovered mainly from non-raft fractions. Upon stimulation of migration, CD44 decreased in raft fractions and increased in non-raft fractions. Raft disruption with methyl-β-cyclodextrin had a similar effect. CD44 and ezrin co-precipitation occurred mainly in non-raft fractions, and was increased in migrating cells. Furthermore, we saw no co-localization of ezrin and the lipid raft marker flotillin-1 in migrating cells. In conclusion, our data suggest that CD44 is released from lipid rafts in order to participate in migratory events involving ezrin binding. As tumour cell migration is an early event in cancer metastasis, further investigation will explore the mechanism and the potential significance for tumour cell dissemination.

2496/B205

Wounding Sheets of Epithelial Cells Activates the Epidermal Growth Factor Receptor through Distinct Short- and Long-Range

Mechanisms.

E. R. Block, J. K. Klarlund; Ophthalmology, University of Pittsburgh, Pittsburgh, PA

Wounding epithelia induces activation of the epidermal growth factor receptor (EGFR), which is absolutely required for induction of motility. ATP is released from cells after wounding; it binds to purinergic receptors on the cell surface, which then triggers activation of the EGFR and the lipid-modifying enzyme Phospholipase D. By modulating phospholipase D activities with isoform-specific siRNA oligonucleotides and monitoring EGFR activation by Western blotting, we show here that ATP activates the EGFR through the phospholipase D2 isoform. Data from novel wounding and microscopic approaches indicate that EGFR activation in cells far (more than 0.3 cm) from wounds is mediated by diffusion of extracellular ATP since activation was abrogated by eliminating ATP in the medium with apyrase. In sharp contrast, activation of the EGFR near wounds was not sensitive to apyrase. After wounding in the presence of apyrase, EGFR activation was blocked by inhibitors of EGFR pro-ligand activity and by inhibitors of Src-family kinases. Time-lapse microscopy revealed that cells exhibit increased motilities near edges of wounds. The increase in motility was not sensitive to apyrase, and apyrase did not detectably inhibit healing of wounds in epithelial sheets. We conclude that wounding activates two distinct signaling pathways that induce EGFR activation and promote healing of wounds in epithelial cells. One pathway signals at a distance from wounds through release of ATP, and another pathway acts locally and is independent on ATP signaling.

2497/B206

A Rho Family GEF Localizes with Actin at the Leading Edge of Cells and Regulates Migration.

J. Bristow[1], M. Sellers[1], D. Majumdar[1], D. Webb[1,2]; [1]Biological Sciences, Vanderbilt University, Nashville, TN, [2]Cancer Biology, Vanderbilt University, Nashville, TN

The Rho family of small GTPases, which includes Rho, Rac and Cdc42, are critical regulators of cell migration through their modulation of the actin cytoskeleton. Activation of the small GTPases is tightly controlled by guanine nucleotide exchange factors (GEFs), but the function of Rho family GEFs in regulating cell migration is not well understood. Here, we show that a new Rho family GEF, which we termed CRAG, localizes with actin at the leading edge of cells where it regulates migration. CRAG increases activation of Cdc42 and Rac by 5-fold and 3-fold respectively, and decreases Rho activation by 2-fold. Knockdown of endogenous CRAG in HT 1080 cells decreased the random migration velocity by approximately 1.5-fold. To further study the function of CRAG in cell migration, we generated stable cell lines expressing low levels of GFP-CRAG (2-fold over endogenous). GFP-CRAG, like endogenous CRAG, localized with actin at the leading edge and increased the velocity of randomly migrating cells by 1.5-fold compared to control cells. In a wound assay, in which cells migrate directionally, the rate of wound-edge migration was increased 2-fold in GFP-CRAG expressing cells compared with control cells. TIRF images show that both the number and size of adhesions are dramatically decreased in GFP-CRAG expressing cells compared to controls. Additionally, the few adhesions found in GFP-CRAG expressing cells are located primarily in the lamella. Analysis of the adhesion turnover rate indicated that adhesions in GFP-CRAG expressing cells assemble approximately 2-fold faster and disassemble 4.4-fold faster than those in control cells, indicating that CRAG significantly increases the rate of adhesion turnover. Inhibitor studies indicate PI3K is an important part of the CRAG pathway. Treatment with the PI3K specific inhibitor, wortmannin, completely abolished the CRAG-mediated effects on cell migration and adhesion turnover. Taken together, our results suggest an important role for CRAG in regulating cell migration through activation of Rho family GTPases, which are critical modulators of actin.

2498/B207

The Cofilin Pathway Determines the Migration Behavior of Metastatic Cancer Cells Both In Vivo and In Vitro.

M. Sidani, J. Wyckoff, W. Wang, J. Condeelis; Anatomy and Structural Biology, Albert Einstein College of Medicine, Bronx, NY

The tumor microenvironment hosts dynamic interactions between tumor cells, macrophages, blood vessels, and extracellular matrix (ECM) fibers. Tumor cell motility is an important determinant of the metastatic potential of tumors. Recent studies indicate that the overall activity of the cofilin pathway proteins, but not that of any single gene within the pathway, determines the invasive and metastatic phenotype of mammary tumor cells. Cofilin F-actin severing ability is important for the regulation of actin dynamics and is negatively regulated by its effector LIMK1. Previously, we showed that siRNA knockdown of cofilin changes the constitutive (in serum) random-walking motility of metastatic mammary cancer (MTLn3) cells into a more persistent and directional motility, concomitant with a decrease in the ability of the cells to change their direction during their migration. These observations led us to investigate the effect of expressing full-length Lim Kinase (F) or its inactive kinase domain (KS) in MTLn3 cells. Analysis of the in vitro motility show that the F cells migrate in a directional manner, and that LIMK expression but not that of KS, inhibits EGF-induced membrane protrusion, chemotaxis and invasion. On the other hand, animals bearing tumors formed from F cells, showed inhibition in invasion and intravasation of tumor cells, and hence increase in their survival. Finally, to study the migration behavior of the F cells in vivo, we employed multiphoton-based intravital imaging (MBIV) of breast tumors formed from either the F or KS cells. Our previous work using MBIV showed that cell orientation toward blood vessels and the ability to cross the vessel wall as an intact cell are important properties of metastatic mammary carcinoma cells. In this context, the motility of cells in relation to their movement on ECM fibers, other cells, and in relation to each other is analyzed.

2499/B208

Intermediate Filaments and the Regulation of Lamellipodia and Ruffled Membranes.

B. Grin[1], B. Helfand[1], L. Chang[2], E. Kuczmarski[1], U. Aebi[3], H. Herrmann[4], R. Goldman[1]; [1]Cell & Molecular Biology, Northwestern University Medical School, Chicago, IL, [2]Harvard University, Boston, MA, [3]University of Basel, Basel, Switzerland, [4]Cancer Research Center, Heidelberg, Germany

Vimentin exists in three distinct structural forms: particulate precursors, short IF (squiggles), and long IF. Using immunofluorescence and live cell imaging of fibroblasts expressing GFP-vimentin, we have observed vimentin particles in the lamellipodium and ruffling membranes of translocating mouse embryonic and 3T3 fibroblasts. Within the lamella there are vimentin particles and squiggles. Longer complex arrays of IF in more proximal regions of the cell body. Within lamellipodia, vimentin precursors are found in close proximity to leading edge markers: VASP, Arp2/3 and cortactin. When the vimentin IF network is disrupted by transfection with a dominant negative vimentin mutant,

pEGFP-vim(1-138), fibroblasts fail to establish a polarized morphology and cannot initiate directed motility, despite the presence of active ruffling membranes and normal appearing microtubule and actin networks. Furthermore, fibroblasts maintained in serum free medium do not have lamellipodia or ruffled membranes, and filamentous IF extend in complex networks to the cell membrane. Under these conditions fasicles of IF are closely associated with focal adhesions. Upon serum addition to these quiescent cells vimentin IF lose their association with focal adhesions as they retract and/or disassemble adjacent to the cell surface. At the same time that membrane ruffling is initiated, there is an increase in the phosphorylation of vimentin at ser-38 suggesting that PKA is involved in the reorganization of vimentin IF and their retrograde movement away from the cell surface. We have developed a peptide derived from the 2B segment of the vimentin central rod domain which disrupts IF assembly in vitro. Microinjection of this peptide disassembles the IF adjacent to the cell surface in serum starved fibroblasts, thereby initiating membrane ruffling in the absence of serum. Taken together, these findings suggest that vimentin IF mechanically stabilize the cell surface and that their local disassembly is directly related to the induction of lamellipodia and the changes in cell shape and polarity required for fibroblast locomotion. Supported by NIGMS.

Cell Motility II (2500 – 2516)

2500/B209

A Genetically Encoded Caged Rac; Divergent Roles of ROCK, PAK and Myosin in Polarized Motility Generated by Localized Rac Activation.

Y. Wu, A. Jaehrig, B. Kuhlman, K. Hahn; University of North Carolina, Chapel Hill, NC

Caged proteins can be activated in cells at precise subcellular locations with subsecond timing. They have proven valuable for defining protein function in dynamic biological processes, but in most cases require covalent modification of proteins, necessitating cell delivery via cumbersome/perturbing techniques such as microinjection. Here we report development of genetically encoded caging using the LOV2 domain of the Avena sativa (oat) phototropin1. Activation is reversible and occurs at 450-500 nm. We fused the small GTPase Rac1 with the LOV2 domain to generate a photoactivatable Rac1 (PA-Rac1). PA-Rac1 can be readily expressed in mammalian cells and can be specifically activated within well-defined regions within microseconds. Switching on Rac1 led to rapid localized actin polymerization. A gradient of Rac1 activity, generated by activating constitutively active or dominant negative Rac1, led to polarized cell movement in opposite directions. Repeated irradiation could be used to control the directionality of cell migration. Rac-induced protrusions were dependent on ROCK and PAK. Inhibition of ROCK, but not MLCK or myosin II, reduced Rac-induced protrusion. In contrast, ROCK inhibition had little effect on the directionality of Rac-induced motility, while MLCK inhibition strongly reduced the ability of Rac gradients to control the direction of migration. This caging approach should be applicable to many other proteins, providing a new tool to explore the spatio-temporal dynamics of signalling in living cells.

2501/B210

Rho-Kinase Contributes to Sustained RhoA Activation through Phosphorylation of P190A RhoGAP.

K. Mori[1,2], M. Amano[1], M. Takehuji[1,2], K. Kato[1,2], T. Nishioka[1], T. Muruhara[2], K. Kaibuchi[1]; [1]Cell Pharmacology, Graduate School of Medicine, Nagoya University, Nagoya, Japan, [2]Cardiology, Graduate School of Medicine, Nagoya University, Nagoya, Japan

RhoA small GTPase is the molecular switch for various extracellular signals, and implicated in a variety of biological functions, including cell contraction, cell migration, cell adhesion, and gene transcription. RhoA regulates these functions through its specific effectors such as Rho-kinase/ROCK/ROK. Rho-kinase phosphorylates myosin phosphatase target protein 1 of myosin phosphatase and thereby inactivates its phosphatase activity, resulting in an increase of phosphorylation of myosin light chain followed by smooth muscle contraction. RhoA is transiently activated by specific vasoconstrictors in vascular smooth muscle cells. Sustained activation of RhoA is induced with high doses of the extracellular signals and is involved in the pathogenesis of cardiovascular diseases such as vasospasm and hypertension. However, it remains largely unknown how prolonged activation of RhoA is induced. In light of this question, we hypothesized that high activation of RhoA/Rho-kinase signaling pathway can inhibit RhoA-specific GAP, leading to sustained RhoA activation. We here show that Rho-kinase, an effector of RhoA, phosphorylated p190A RhoGAP, a best-characterized RhoA-specific GAP, at Ser1150 and attenuated the activity of p190A RhoGAP in intact cells. Binding of Rnd to p190A RhoGAP is thought to enhance RhoGAP activity and inactivate RhoA. We also found that phosphorylation of p190A RhoGAP by Rho-kinase impaired Rnd binding, suggesting that Rho-kinase counteracts p190A RhoGAP activity. Stimulation of vascular smooth muscle cells with endothelin-1 (ET-1) provoked phosphorylation of p190A RhoGAP in a Rho-kinase dependent fashion. Moreover, the phosphomimic mutation of p190A RhoGAP weakened Rnd-binding and RhoGAP activity. These results suggest that ET-1 induced Rho-kinase activation and subsequent phosphorylation of p190A RhoGAP, thereby constituting the positive feedback loop to prolonged RhoA activation.

2502/B211

Labile Function of the Small GTPase Rap1 in Cell Adhesion and Movement Depends on the Activity of the IGF-I Receptor.

M. A. Guvakova[1,2], W. Lee[2], D. K. Furstenau[1]; [1]Surgery, University of Pennsylvania, Philadelphia, PA, [2]Microbiology, University of Pennsylvania, Philadelphia, PA

The Ras family GTPase Rap1 is an essential regulator for adhesion receptors, actin dynamics, and cell migration. The mechanisms by which Rap1 controls motile responses in the epithelial cells remain unknown. In fact, current view is that activation of Rap1 tightens cell-cell junctions and thus may have anti-metastatic effects in cancer. Normal and cancer cells share common, and perhaps evolutionarily conserved, motility machinery activated by insulin-like growth factor I (IGF-I). In this study, we sought to investigate whether IGF-I regulated Rap1 and if so, to examine the functional consequences of Rap1 regulation to the integrity of epithelial contacts. We found that autophosphorylation and internalization of the IGF-I receptor (IGF-IR) triggered a spike of Rap1 GTP-loading in conjunction with disruption of epithelial contacts. We used an in vivo tool, a fusion of enhanced green fluorescent protein (EGFP) to the Rap1 binding domain (RBD) of RalGDS and showed that endogenous Rap1 activated by IGF-I is recruited to the edges of lamellipodia. By time-lapse video microscopy, we tracked

localization of the EGFP-WTRap1 (wild type), -N17Rap1 (constitutively inactive mutant), and -V12Rap1 (constitutively active mutant) in live cancer cells. We showed that sustained activation of Rap1 by itself was insufficient to trigger disruption of epithelial contacts or to prevent the disruption of epithelial contacts and loss of E-cadherin induced by IGF-I. Activity of endogenous Rap1 appeared to be necessary for the cell movement, as inactivation of Rap1 by overexpressed RapGAP decreased the intracellular level of actin fibers and blocked lamellipodia formation. These studies implicate Rap1 in the control of cell adhesion and movement via regulation of the actin cytoskeleton. Furthermore, quantitative immunohistochemistry analysis of surgical specimens revealed significantly higher protein levels of IGF-IR (p=4.32E-07) and its downstream target Rap1 (p=6.23E-04) in invasive breast carcinoma (n=32) compared with normal and benign (n=23) breast tissue.

2503/B212

GRASP/tamalin Mediated Crosstalk between ARF6 and Rac.

D. T. White, L. Santy; Department of Biochemistry and Molecular Biology, Pennsylvania State University, University Park, PA

Cellular migration requires precise coordination of alterations in cellular signaling and architecture. Small GTPases of the Rho and ARF families regulate these processes. We have previously shown that activation of ARF6 by the GEF ARNO profoundly changes the morphology and behavior of epithelial MDCK cells. MDCK cells over-expressing ARNO form large fan-shaped lamellipodia and become highly motile. This enhanced motility requires activation of ARF6 and the subsequent downstream activation of Rac1. The Dock180/Elmo complex mediates Rac activation downstream of ARNO and ARF6. These two GEF proteins may be working together to coordinate the activation of ARF6 and Rac1 at the leading edge of migrating cells. We have found that expression of ARNO is significantly more efficient than activated ARF6 mutants at inducing robust activation of Rac1. This suggests that ARNO may precisely localize ARF6 activity for subsequent activation of Rac. Additionally, a truncation mutant of ARNO lacking the N-terminal coiled-coil domain fails to induce motility. This mutant activates ARF6 to normal levels but is deficient at inducing activation of Rac1. We hypothesize that protein-protein interactions mediated by the coiled-coil domain of ARNO are critical for properly localizing activation of ARF6 for crosstalk with Dock180 and activation of Rac1. We are currently investigating the involvement of three proteins known to bind to this domain of ARNO in the regulation of epithelial motility. These are the scaffolding proteins GRASP/tamalin, Cybr and IPCEF. Knockdown of GRASP inhibits the ability of ARNO to induce Rac activation. We are investigating the possibility that GRASP mediates the formation of a complex containing both ARNO and Dock180.

2504/B213

ACAP4-Ezrin Interaction Links EGF Signaling to Arf6 Activation and Breast Cancer Invasion.

F. Wang, X. Ding, X. Yu, P. Xia, D. Wang, H. Deng, Z. Guo, X. Yao; Cell Biology, Cellular Dynamics, Hefei, China

Epidermal growth factor (EGF) receptor (EGFR) signalling is implicated in tumour invasion and metastasis. However, whether there are EGFR signalling pathways specifically used for tumour invasion still remains elusive. Overexpression of Arf6 correlates with and is crucial for the invasive phenotypes of different breast cancer cells. Our recent proteomic study has identified a novel Arf6 GTPase-activating protein ACAP4 (Mol. Cell Proteomics 2006. 5, 1437-1448). Here we identify the mechanism by which Arf6 is activated by an ACAP4-ezrin interaction to induce tumour invasion. Our biochemical studies identified that ACAP4 interacts with EGF-phosphorylated ezrin in vitro and in vivo. This phosphorylation-regulated ezrin-ACAP4 interaction is mediated by its pleckstrin homology domain, which activates Arf6 GTPase activity. Using siRNA-mediated knock-down approach, we found that ACAP4 is responsible for the invasive activity of MDA-MB-231 breast cancer cells while the other ArfGAPs are not. Overexpression of ACAP4, together with Arf6, caused non-invasive MCF7 cells to become invasive, which was dependent on ezrin phosphorylation. Importantly, ACAP4 was expressed in primary breast ductal carcinomas, and was preferentially co-expressed with phosphorylated ezrin in the malignant cases. Our results indicate that ACAP4 links EGFR signalling to Arf6 activation to induce invasive activities of some breast cancer cells, and hence may contribute to their metastasis and malignancy.

2505/B214

Differential Control of Phosphorylation of Grb7 by FAK in Regulating Cell Migration and Proliferation.

P. Chu[1], C. Hsu[1], J. Guan[2], T. Shen[1]; [1]Department of Plant Pathology and Microbiology, National Taiwan University, Taipei, Taiwan, [2]Department of Internal Medicine-Division of Molecular Medicine and Genetics Dept of Cell and Developmental Biology, University of Michigan Medical School, Ann Arbor, MI

Growth factor receptor-bound protein 7 (Grb7) is a src-homology 2 (SH2) containing adaptor protein that is found to interact with FAK to promote cell migration. To further elucidate the signaling event of FAK-Grb7 complexes in promoting cell migration and other cellular functions, we examined the Grb7's tyrosine phosphorylation site(s) by FAK using an in vivo mutational analysis and an in vitro mapping approach. We found that two out of 12 tyrosine residues, Y188 and Y338, within Grb7 enable phosphorylated by FAK and both of which are crucial for cell migration in an integrin-dependent manner. In addition, Y188F, Y338 or R239L also exhibited an inhibitory ability on cell growth and survival comparing with wild-type Grb7. In contrast, employment of lentiviruses expressing Grb7 small interfering RNA (siRNA) to proceed gene knockdown could reverse the migratory effect caused by these Grb7 and its mutants. Furthermore, phosphorylation of ERK but not p38 was elevated by Grb7 phosphorylated by FAK, indicating that a novel pathway FAK-Grb7-ERK involves in cell migration in response to integrin activation. On the other hand, increased phosphorylation of AKT was responsible for FAK/Grb7-mediated cell proliferation. Our results suggested a differential control of Grb7's phosphorylation by FAK in regulating cell migration and proliferation through either ERK or AKT, respectively. Key words: Grb7, FAK, cell migration, cell proliferation

2506/B215

Caspase-8 Is an Essential Component of EGF-Induced Erk Pathway Activation.

D. Finlay, A. Howes, K. Vuori; Cancer Center, Burnham Institute for Medical Research, La Jolla, CA

Caspase-8 is a well characterized protease involved in the "extrinsic pathway" of apoptosis. Recent work, however, suggests the protein has additional non-apoptotic roles in cell adhesion/ motility and related downstream signaling events. The objective of our studies is to investigate what role caspase-8 plays in epidermal growth factor-(EGF) induced cell motility and associated signaling events. We demonstrate that caspase-8 is essential for the classical Erk pathway signaling associated with EGF. We show that this role is independent of the protein's catalytic activity and can in fact be re-capitulated using only caspase-8's pro-domain "death effector domains" (DEDs). In addition we identify specific residues within the first DED of caspase-8 that are required for EGF-induced Erk pathway activation and we demonstrate that point mutants of this region are also refractory to EGF-induced cell motility. We also provide evidence to suggest that this promulgation of EGF-induced Erk pathway activity by caspase-8 is due to its ability to associate with a protein complex containing Src. Indeed, the necessity of caspase-8 for EGFR/ Src signaling may explain why caspase-8 is rarely deleted or silenced in cancers.

2507/B216

P53 Suppresses Src-Induced Podosome/Invadopodia Formation, Cell Motility and Invasion by Deactivation of Stat3.

P. Mooney, U. Mukhopadhyay, R. Eves, L. Xuhong, A. S. Mak; Queen's University, Kingston, ON, Canada

The mechanism by which Stat3, a pro-metastatic factor, enhances cell migration and invasion is poorly understood. Here we show that Stat3 acts as a downstream effector of Src in affecting cytoskeletal rearrangements in vascular smooth muscle and NIH3T3 cells leading to the formation of podosome/invadopodia, which are important for cell motility and invasion. shRNA-mediated knockdown of Stat3 down regulates Src-induced podosome/invadopodia formation, extracellular matrix degradation, cell migration and invasion. We also found that p53, the tumor suppressor protein, strongly suppresses Src-induced invasive phenotypes. Ectopic expression of wild type p53 or activation of endogenous p53 by Adriamycin negatively affects Src-induced podosome/invadopodia formation, extracellular matrix degradation, cell migration and invasion. In addition, over-expression of p53 or activation of p53 with Adriamycin reduces nuclear localization of Stat3 as well as decreases the active form of Stat3 (pY705-Stat3). In contrast, expression of constitutively active Stat3 largely abrogates the suppressive effect of p53 on Src-induced invasive phenotypes. This data suggests that p53 may play a role in the attenuation of Src-induced cell migration and invasion by down regulating Stat3 in both vascular smooth muscle and NIH3T3 cells.

2508/B217

Bacillus Anthracis Toxins Paralyze Neutrophil Actin-based Motility.

S. Guilmain[1], R. During[1], W. Li[1], C. Quinn[2], W. Tang[3], F. Southwick[1]; [1]Medicine, Infectious Diseases, University of Florida, Gainesville, FL, [2]CDC, Atlanta, GA, [3]University of Chicago, Chicago, IL

Inhalation anthrax results in high-grade bacteremia and shock that is accompanied by a blunted rise in the peripheral neutrophil count and a paucity of neutrophils in infected pleural fluid. Edema toxin (ET) is one of the major Bacillus anthracis virulence factors and consists of the adenyl cyclase, edema factor (EF) and protective antigen (PA). Relatively low concentrations of ET (100-500ng/ml) significantly impair human neutrophil chemokinesis, chemotaxis and the ability to polarize. These changes are not accompanied by a significant change in CD11/CD18 receptor expression with ET or LT alone, however the combination of the two toxins causes a significant decrease in receptor expression. Toxin treatment is also associated with a 30% reduction in FMLP-stimulated F-actin content. ET treatment also reduces the speed of Listeria monocytogenes intracellular actin-based motility by 30%, and decreases actin filament tail length by 50% in HeLa cells. These defects in actin assembly are accompanied by >50 fold increase in intracellular cyclic-AMP and a >4 fold increase in the phosphorylation of protein kinase A (PKA). Our results demonstrate that ET and the combination of ET + LT is able to suppress human neutrophil migration and polarity, and these defects are a consequence of a decrease in the adhesion receptor and a reduction in actin assembly, that in turn may be mediated by high intracellular levels of cyclic-AMP and the phosphorylation of PKA.

2509/B218

Camp Signaling Affects Neuronal Firing Properties and Filopodial Dynamics of Neuronal Growth Cones.

L. Zhong, L. Artinian, V. Rehder; Biology Department, Georgia State University, Atlanta, GA

Cyclic adenosine monophosphate (cAMP) serves as an important second messenger and is known to regulate neurite outgrowth in vivo and in vitro. The mechanism of cAMP signaling that determines growth cone motility and filopodial dynamics in particular is largely unknown. Here, we report that elevation of cAMP by the adenylate cyclase activator forskolin or the cAMP analog 8-bromo-cAMP caused a rapid and transient elongation in filopodial length and a reduction in the number of filopodia in identified *Helisoma* B5 neurons. Forskolin also elicited an elevation of the free intracellular calcium concentration ($[Ca^{2+}]_i$) in treated growth cones, which was correlated with the morphological change in filopodial dynamics. Pretreatment with $CdCl_2$, a blocker of voltage gated calcium channels, significantly inhibited the increase in the $[Ca^{2+}]_i$ in the growth cones, and also blocked filopodial elongation induced by forskolin. Furthermore, whole-cell current-clamp recordings of the electrical activity in B5 neurons showed that the elevation of cAMP resulted in a significant broadening of action potentials and a decrease in their peak amplitude. $CdCl_2$ treatment reversed the cAMP-induced changes in the action potential, suggesting that cAMP might act via modulation of calcium channels that contribute to the broadening of the action potential. By linking cAMP signaling and modulation of the voltage-gated calcium channel activity in B5 neurons, we suggest that cAMP signaling affects growth cone motility via modulation of voltage-gated calcium channels during neuronal pathfinding. cAMP could be acting directly in the growth cone or at a distant location affecting growth cone motility via its modulation of action potentials, which would propagate to the growth cone and result in an increase in $[Ca^{2+}]_i$. --This work was supported in part by NSF grant 0343096 to VR.

2510/B219

The Role of GDI in Spatio-temporal Regulatin of Cdc42 Activity.

M. Sabouri-Ghomi[1], L. Hodgson[3], G. Danuser[1], K. Hahn[2]; [1]Cell Biology, the Scripps Research Institute, La Jolla, CA, [2]Pharmacology, University of North Carolina at Chapel Hill, Chapel Hill, NC, [3]Anatomy and Structural Biology, the Albert Einstein College of Medicine of Yeshiva University, New York, NY

Rho family GTPases are important signaling molecules implicated in the regulation of cell polarization and motility. Their activation is tightly coordinated in space and time by upstream regulators including guanine-nucleotide dissociation inhibitors (GDI), which sequester inactive GTPases in the cytosol and mediate localization of activated GTPases in the plasma membrane. Previous work in our labs has established the sequence of Rho GTPase activation during cell protrusion on a time scale of seconds and with single micron spatial resolution. However, which factors are implicated in this control, and specifically whether GDI - GTPase interactions are critical to it, has been unknown. To study the regulatory effect of GDI on GTPases we chose Cdc42. We developed a novel, genetically-encoded fluorescent biosensor to directly visualize the endogenous GDI binding to Cdc42 in living cells and in real time. To monitor GDI-Cdc42 interactions and the activation state of Cdc42 simultaneously, we re-engineered the previously reported activation biosensor for endogenous Cdc42, based on dyes. Our data shows that GDI association with Cdc42 is negatively correlated with both Cdc42 activity at the leading edge and protrusion, while Cdc42 correlates positively with protrusion. Statistical analysis of the correlation functions reveals that maximum binding between GDI and Cdc42 is reached during the retraction phase, localized at membrane ruffles. Approximately 40 seconds after maximum GDI-Cdc42 association is reached, GDI-Cdc42 complexes dissociate accompanied by maximal Cdc42 activity. This defines a mechanism for our earlier and surprising finding that Cdc42 lags the time point of maximal edge advancement. To test the robustness of these predictions we perturbed Src kinases upstream of GDI. Our results showed that perturbation of GDI phosphorylation disrupts both the timing and localization of GDI binding/unbinding with Cdc42. These defects in signaling are directly affecting the protrusion efficiency of cells.

2511/B220

Regulation of Neural Precursor Cell Migration by Sphingolipids and Its Critical Role for Mouse Brain Development.

G. Wang, K. Krishnamurthy, Y. Chiang, S. Dasgupta, E. Bieberich; Medical College of Georgia, Augusta, GA

Ceramide is a sphingolipid known to induce apoptosis. We show here that it also regulates neural precursor cell (NPC) migration in vitro and that it is critical for brain development in vivo. Ceramide (C16:0 and C18:1) and the ceramide analog N-oleoyl serinol (S18) stimulate migration of NPCs in scratch (wounding) migration assays. Sphingolipid depletion by inhibition of de novo ceramide biosynthesis, or ceramide inactivation using an anti-ceramide antibody, obliterates NPC motility, which is restored by ceramide or S18. These results suggest that ceramide is crucial for NPC motility. Wounding of the NPC monolayer activates neutral sphingomyelinase indicating that ceramide is generated from sphingomyelin. In membrane processes, ceramide is co-distributed with its binding partner atypical PKζ/λ (aPKC), and Cdc42, α/β-tubulin, and β-catenin, three proteins involved in aPKC-dependent regulation of cell polarity and motility. Sphingolipid depletion by myriocin prevents membrane translocation of aPKC and Cdc42, which is restored by ceramide or S18. These results suggest that ceramide-mediated membrane association of aPKC/Cdc42 is important for NPC motility. In vivo, sphingolipid depletion leads to ectopic localization of mitotic or post-mitotic neural cells in the embryonic brain, while S18 restores the normal brain organization. In summary, our study provides novel evidence that ceramide is critical for NPC motility and polarity in vitro and in vivo.

2512/B221

Protein Kinase a Regulates 3' Phosphatidylinositol Dynamics during PDGF-induced Membrane Ruffling and Chemotaxis.

P. Deming[2], S. L. Campbell[1], L. C. Baldor[1], A. K. Howe[1]; [1]Pharmacology, University of Vermont, Burlington, VT, [2]Medical Laboratory and Radiation Sciences, University of Vermont, Burlington, VT

Spatial regulation of the cAMP-dependent protein kinase (PKA) is required for chemotaxis in fibroblasts, however the mechanism(s) by which PKA regulates the cell migration machinery remain largely unknown. Here we report that one function of PKA during PDGF-induced chemotaxis is to promote membrane ruffling by regulating phosphatidylinositol 3,4,5-trisphosphate (PIP3) dynamics. Cells lacking PKA activity exhibit dramatically altered membrane dynamics and attenuated formation of peripheral membrane ruffles in response to PDGF. Inhibition of PKA also significantly decreased the number and size of PIP3-rich membrane ruffles in response to uniform stimulation with PDGF and to gradients of PDGF. This defect was quantified using a newly-developed method, based on computer vision edge-detection algorithms, for quantification of membrane ruffling. PKA-inhibited cells also displayed a marked attenuation in the bulk accumulation of PIP3 induced by treatment with PDGF. The deficits in PIP3 dynamics correlated with a significant inhibition of growth factor-induced Rac activation in PKA-inhibited cells. Moreover, the expression of a constitutively-active Rac allele was able to rescue the defect in membrane ruffling and restore the localization of a fluorescent PIP3 marker to membrane ruffles in PKA-inhibited cells. Finally, simultaneous inhibition of PKA and Rac had an additive inhibitory effect on growth factor-induced production of PIP3-rich ruffles. These data demonstrate that membrane dynamics and the distribution of PIP3/3' phosphatidylinositol (3'PI) lipids represent major targets for PKA in PDGF-induced chemotactic events.

2513/B222

pH Sensors Regulating Cell Migration.

B. Webb[1], F. Milletti[2], M. Jacobson[2], D. Barber[1]; [1]Cell and Tissue Biology, UCSF, San Francisco, CA, [2]Pharmaceutical Chemistry, UCSF, San Francisco, CA

A conserved hallmark of most metastatic cells regardless of their tissue origin or genetic background is an elevated intracellular pH (pHi) that is 0.3-0.4 units higher than normal cells. Additionally, emerging evidence indicates that increased pHi is a conserved mechanism necessary for directed cell migration. To identify regulators of pH-dependent cell migration we developed a strategy to identify growth factor-stimulated pH sensors or proteins that have activities or ligand-binding affinities that are sensitive to physiological increases in pHi. We asked what proteins show changes in phosphorylation or membrane association in a pH-dependent manner by using fibroblasts expressing a wild-type Na-H exchanger NHE1 (WT cells) that have increased pHi with PDGF or expressing an inactive NHE1-E266I that lacks H+ efflux (E266I cells) and have no change in pHi with PDGF. The abundance of 4 proteins was markedly different in membrane fractions of growth factor-stimulated WT cells compared with quiescent cells but unchanged abundance in E266I cells. Our data suggested that of the 85kD protein was the p85 subunit of PI3K. Immunoblotting proteins co-precipitating with anti-phosphotyrsoine antibodies confirmed a 2-fold increase in abundance of p85 with PDGF in WT cells compared with E266I cells. Additionally, increased PI3K activity with PDGF in WT cells was attenuated 4-fold in E266I cells. Molecular modeling of the carboxy-terminal SH2 domain (C-SH2), which contains a key His

residue (His706) required for binding activated PDGF receptors, suggests a pH-dependent regulatory mechanism: protonation of His706 destabilized α-helix B, leading to its partial unwinding. We predict that deprotonation of His706 with increased pHi induces conformational changes in C-SH2 and increased affinity of PI3K for its tyrosine phosphorylated ligands, including the PDGF receptor. Our data suggest that PI3K may be a previously unrecognized pH sensor regulating pH-dependent cell migration and metastasis.

2514/B223

Directional Cell Motility Regulated by a FAK-p120RasGAP-p190RhoGAP Complex.

A. Tomar[1], S. Lim[1], Y. Lim[2], D. Schlaepfer[1]; [1]Moores Cancer Center, UCSD, La Jolla, CA, [2]Mogam Research Institute, Yongin, South Korea

Directional motility is a complex process requiring spatio-temporal integration of signals regulating cytoskeletal changes and the establishment of an anterior-posterior or polarized cell axis. Focal adhesion kinase (FAK) promotes cell migration, but a molecular role for FAK in promoting cell polarity remains undefined. Here, using wound healing motility and Golgi reorientation analyses, we show that FAK is required for polarized fibroblast, endothelial, and carcinoma cell motility. Polarity defects of FAK /- mouse embryo fibroblasts (MEFs) are rescued by a focal adhesion (FA) targeted Pyk2-FAK-CT chimera and by re-expression of wild-type and FAK phosphorylation site mutants (F861 and F925), but not FAK mutated at Y397 nor catalytically-inactive FAK (R454). FAK activity is required for fibronectin (FN)-stimulated p190RhoGAP (p190A) tyrosine phosphorylation, p190A-p120RasGAP complex formation, and inhibition of RhoA activity. Over-expression of GAP-inactive (R1283A) or phosphorylation site mutated (Y1087F, Y1105F) p190A in FAK+/+ MEFs blocks cell polarity. Knockdown of p120RasGAP prevents FN-stimulated FAK-p190A complex formation, p190A tyrosine phosphorylation, and FAK+/+ MEF polarity. Further, although p190A is tyrosine phosphorylated and complexed with p120RasGAP in Src-transformed FAK-/- MEFs, FAK is required for FA-associated p190A localization and cell polarity. Our studies show that FAK expression and activity facilitate the recruitment and stabilization of a p120RasGAP-p190A complex at leading edge FAs connected to the regulation of directional cell movement.

2515/B224

Signaling Vector Analysis (SVA) of Intracellular Pathways Governing Cell Migration.

A. Melvin, M. Weiger, J. Haugh; Chemical & Biomolecular Engineering, North Carolina State University, Raleigh, NC

Cell migration is characterized by cyclic protrusion, adhesion, and contractile events that are regulated through a complex network of intracellular pathways that are often directed by soluble and adhesion-based signals. Previous work has established that regions of membrane protrusion in fibroblasts are localized hot spots of phosphoinositide 3-kinase (PI3K) signaling, which in the context of PDGF-stimulated chemotaxis are apparently reinforced when cells migrate up-gradient. These observations, which have more recently been extended to include the contexts of fibroblast spreading and random migration on adhesive surfaces, suggest a hypothetical mechanism by which migration of fibroblasts and similar cell types is spatially directed. To explore these ideas further and in a quantitative fashion, we have developed signaling vector analysis (SVA), a novel image analysis protocol that relates the spatial pattern of intracellular signaling to aspects of cell movement. Specifically, SVA relates the orientations, intensities, and lifetimes of signaling hot spots to cell speed and persistence. Using this approach in conjunction with total internal reflection fluorescence microscopy, we compare the relationships between PI3K signaling and migration behavior in the contexts of random fibroblast migration and PDGF-stimulated chemotaxis in a microfluidic device.

2516/B225

A Role for TGF-beta in Zebrafish Keratocyte Motility.

B. Tan, K. Cooper, E. Hull; Biomedical Sciences, Midwestern University, Glendale, AZ

The fish keratocyte is a model system for the study of the mechanics of cell motility, because of the rapid, smooth gliding motion that is characteristic of the movement of these cells. However, little characterization of the molecules and signal transduction pathways involved in this unique mode of movement has been done. As TGF-beta plays multiple roles in primary human keratinocyte cell migration and differentiation, we investigated the possible involvement of TGF-beta in fish keratocyte migration. We found that transcription of TGF-beta1 and TGF-betaRII genes, measured by microarray analysis and qRT-PCR, increased during primary explant culture of these cells. As measured by ELISA, the levels of TGF-beta in the cell culture media increase during the first 24-hours of explant culture in serum free and low-serum media. Furthermore, addition of TGF-beta to the culture media increased the persistence, or net distance migrated per unit time, for keratocytes cultured in serum free and low-serum media. Together, these data indicate that expression of TGF-beta and its receptor are up-regulated during explant culture and that TGF-beta promotes fish keratocyte migration.

Cytoskeletal Organization III (2517 – 2537)

2517/B226

Movement of GTPase Activity Zones during Xenopus Oocyte Wound Healing.

B. Burkel, W. Bement; University of Wisconsin, Madison, WI

The small Rho GTPases, Rho and Cdc42, are key regulators of the cytoskeleton and organize biological activities in numerous cellular processes including wound healing. During single-cell wound healing, Rho and Cdc42 form discrete zones of activity around the wound perimeter. Rho activity is primarily confined to the wound edge where it is circumscribed by a zone of Cdc42 activity. These concentric zones move forward with remarkable precision that parallels the movement of the contractile ring that closes cellular wounds. It is unclear how this precise forward movement is accomplished, but understanding the mechanism by which the zones of GTPase activity move forward is not only valuable for understanding the contractile process they are regulating, it also provides insights into how the GTPases themselves are activated and organized. Some clues to this mechanism have come from observations that contractile rings will continue to close in the absence of myosin 2-based contractility and that quantification of GTPase fluorescence decay rates show differences in lifetime activity

within their respective zones. As a result, we propose a novel signaling mechanism that does not depend on transport of the active molecules but instead on the differential activation and inactivation of the GTPases.

2518/B227

Structure/Function Studies on IpgB2, a RhoA Mimic from Shigella.

K. Gold, G. D. Longmore, D. Sept; Washington University in St. Louis, Saint Louis, MO

Significant cytoskeletal rearrangements are required for bacterial invasion of host epithelial cells. Rho GTPases are directly involved in this cytoskeletal reorganization, and bacteria have evolved some truly remarkable strategies to hijack the relevant Rho-dependent signaling cascades. Most bacterial effectors that impact Rho GTPase signaling act as upstream Rho effectors; however, recent studies have identified a new class of bacterial proteins that can directly mimic and effectively subvert the Rho GTPases themselves [1]. *Shigella flexneri*, an agent of bacillary dysentery, encodes two such Rho GTPase mimics, IpgB1 (a RhoG mimic) and IpgB2 (a RhoA mimic) [1,2]. These proteins can bind to the same downstream Rho effectors and elicit some of the same cytoskeletal phenotypes, but what makes this mimicry truly remarkable is that these bacterial proteins mimic Rho function while lacking sequence similarity to, and the biochemical attributes characteristic of Rho GTPases: they do not require GTP to function, and are impervious to Rho GTPase-activating-proteins (GAPs), guanine-nucleotide-exchange factors (GEFs), and inhibitors [1,2]. We are interested in identifying the mechanism of action of IpgB2: specifically, how it is capable of interacting with Rho effectors, mDia and ROCK [1], given the marked biochemical differences between IpgB2 and RhoA. We predicted the structure of IpgB2 and the composition of the ROCK/mDia:IpgB2 binding interfaces computationally, and proceeded to test our computational predictions in the lab via site-directed mutagenesis and immunofluorescence, using stress fiber formation as a readout (present when wildtype IpgB2 or constituently active RhoA is expressed in these cells). We found that when we made mutations to IpgB2 residues at the predicted binding interface, we saw the abrogation of stress fiber formation in U2OS cells. These results provide insight into how IpgB2 is able to subvert RhoA-dependent signaling pathways to aid in *Shigella* invasion of colonic epithelial cells. 1) N. M. Alto et al., *Cell* **124**, 133 (2006). 2) M. Ogawa, Y. Handa, H. Ashida, M. Suzuki, C. Sasakawa, *Nat Rev Microbiol* **6**, 11 (2008).

2519/B228

Cdk5 Activity Regulates Cytoskeletal Organization by Controlling Rho-Dependent Myosin Phosphorylation.

B. Tripathi, C. Y. Gao, P. Zelenka; National Eye Institute, NIH, Bethesda, MD

Phosphorylation of myosin regulatory light chain (MRLC) at Thr18/Ser19 regulates myosin-dependent cytoskeletal contraction, essential for cell adhesion, migration, and division. In epithelial cells, Rho kinase (ROCK) is primarily responsible for this phosphorylation. We have shown that cyclin dependent kinase 5 (Cdk5), a Ser/Thr kinase, regulates cytoskeletal organization in epithelial cells, raising the possibility that Cdk5 might affect Rho-dependent myosin diphosphorylation and contraction. To test this, we examined human lens epithelial cells spreading on fibronectin. Rho activation, MRLC diphosphorylation, and Cdk5 kinase activity increased in parallel from 15 to 120 min after plating. MRLC diphosphorylation was accompanied by a characteristic transition from convex to concave cell boundaries, indicating myosin contraction. Immunofluorescence showed that Cdk5 and its activator, p35, co-localized with diphosphorylated MRLC on contracting stress fibers. Inhibiting Cdk5 activity with a pharmacological inhibitor (olomoucine), siCdk5, or dominant negative GFP-dnCdk5(D144N), significantly reduced the level of diphosphorylated MRLC at 120 min and reduced the percentage of cells that assumed the stellate configuration. Immunostaining confirmed that diphosphorylated MRLC was significantly reduced throughout the cell and revealed striking changes in stress fiber organization with almost complete loss of radial stress fibers. Since MRLC lacks a favorable site for phosphorylation by Cdk5, we tested whether Cdk5 affects Rho-GTP formation or ROCK activation in the upstream signaling cascade. Both olomoucine and siCdk5 significantly reduced Rho-GTP formation and ROCK activation in spreading cells. Since Rho-GTP is required for actin polymerization of radial stress fibers as well as for myosin contraction, an effect on either could be responsible for their loss. Therefore, we tested the effect of inhibiting Cdk5 activity after stress fibers were fully formed. Even under these conditions, Cdk5 inhibition significantly reduced MRLC diphosphorylation and caused preferential loss of radial stress fibers, demonstrating that Cdk5 activity is required for Rho-dependent myosin diphosphorylation and cytoskeletal contraction.

2520/B229

Rhoa/ROCK Pathway and Calcium Signal - Balance of Crosstalk.

P. Krzeminski[2], D. Suplat[1], A. Wasik[1], J. Jaworski[3], P. Pomorski[1], W. Klopocka[2]; [1]Cell Biology, Nencki Institute of Experimental Biology, Warsaw, Poland, [2]Cell Biochemistry, Nencki Institute of Experimental Biology, Warsaw, Poland, [3]Neurobiologu, International Institute of Molecular and Cellular Biology, Warsaw, Poland

There are two basic signaling pathways, regulating the functional state of actin cytoskeleton, namely RhoA and calcium pathways. In current study, we have investigated the crosstalk of those pathways. To evaluate molecular mechanism of this crosstalk, activity of two actin-binding proteins - cofilin and myosin II was studied. Their phosphorylation level was measured by Western blot and immunofluorescence analysis. We have observed, that in glioma C6 cells, stimulation of $P2Y_2$ nucleotide receptor induces cofilin and myosin II phosphorylation. The myosin light chain phosphorylation stimulate actomyosin activity and cell contractility while cofilin phosphorylation inactivate this protein and stabilizes actin network. Since it is known, that activation of this receptor leads to activation of RhoA, we have checked, using ROCK inhibitor, if this is the only active pathway. We have found that effect of ROCK inhibitor can be compensated by calcium signal. The effect observed on the biochemical level is visible also in the cell morphology. To control this effect, we studied changes of 3D cell shape by confocal microscopy. Inhibition of ROCK kinase leads to statistically significant rounding of the cell, measured in volume to area ratio as well as cell height. These changes are also compensated by induction of calcium signal. We suggest that activation of PLC and PIP_2 hydrolysis accompanying formation of G-coupled receptor initiated calcium signal are responsible for inactivation of cofilin. MLC phosphorylation is directly evoked by calcium signal due to activity of calmodulin dependent kinase (MLCK). Concluding, the activation of $G_{\alpha q}$ coupled receptors evoke two parallel signaling pathways which may act synergistically or compensate the inhibition of one of them by the activation of other.

2521/B230

Neuregulin-1 Regulates Cytoskeletal Reorganization and Cell Migration in PDGF-BB Stimulated Rat Vascular Smooth Muscle Cells.

V. S. Cannon, A. Hardin, B. D. Ford; Neuroscience Institute, Morehouse School of Medicine, Atlanta, GA

Vascular smooth muscle cell differentiation as manifested by biosynthetic and contractile behaviors are critical to normal vascular development. However vascular smooth muscle cells exhibit varied responses to vascular injury and surgical interventions, which include a shift from quiescent and contractile to proliferative and migratory, leading to vascular occlusion, neointimal formation and restenosis. Factors that inhibit proliferation and migration of VSM may be useful for prevention and therapy in vascular disease. The signal transduction pathways that mediate phenotypic switching in vascular smooth muscle are being defined. Some of these pathways include Ras-mitogen activated protein kinase, Rho, and NO pathways. Neuregulin-1, a family of glycoproteins that activate ErbB 2-4 receptor tyrosine kinases can mediate the proliferation, differentiation, and survival of a number of cell types, has the potential to mediate the VSM cell response to vascular injury. Neureguin-1 is expressed in vascular endothelial cells and its receptors are localized to the underlying smooth muscle cells. Work in our lab has begun to illuminate the role of NRG-1 on vascular function after balloon injury. Although low in uninjured arteries, NRG-1 expression was upregulated following balloon injury, and that administration of NRG-1 two weeks post injury resulted in a 50% reduction in neointimal formation. Furthermore, rat VSM cells pretreated with NRG-1 for 24 hours, and then stimulated with PDGF showed that NRG-1 significantly decreased PDGF-stimulated proliferation and migration. Here we continue to test the hypothesis that NRG-1 prevents neointimal formation following vascular injury by regulating the phenotypic and proliferative changes observed in vascular lesion formation. We demonstrate that NRG-1 reduces PDGF-BB stimulated migration and reduced the conversion of globular to filamentous actin, stress fibers and reduced the formation of membrane ruffles, filopodia, and lamellipodia.

2522/B231

SWF1p, a Member of the DHHC-CRD Palmitoyltransferase Family, Regulates the Actin Cytoskeleton and Polarized Secretion Independently of Its DHHC Motif.

S. A. Dighe[1], K. G. Kozminski[1,2]; [1]Biology, University of Virginia, Charlottesville, VA, [2]Cell Biology, University of Virginia School of Medicine, Charlottesville, VA

Rho and Rab family GTPases play a key role in cytoskeletal organization and vesicular trafficking, but the exact mechanisms by which these GTPases regulate polarized cell growth are incompletely understood. A synthetic genetic interaction screen in *S. cerevisiae* for genes that interact with *CDC42*, which encodes a conserved Rho GTPase required for polarized cell growth, found *SWF1* (spore wall formation)/*PSL10* (profilin synthetic lethal)(Kozminski *et al*. 2003. *MBoC* 14:4958). *SWF1* encodes a predicted multi-pass membrane protein of the DHHC-CRD palmitoyltransferase family. Consistent with the Swf1p-dependent palmitoylation that blocks SNARE degradation (Valdez-Taubas & Pelham. 2005. *EMBO J* 24:2524), we found *SWF1* deletion caused secretion defects and the accumulation of 80-100nm vesicles within buds. With an antibody against the C-terminal region of Swf1p, we show this region of Swf1p localized to cortical actin patches and along actin cables. This localization pattern was distinct from that of Cdc42p, suggesting that Swf1p and Cdc42p do not interact directly, although Cdc42p localization at sites of polarized growth, after bud emergence, depended upon Swf1p. Deletion of *SWF1* also resulted in mis-organization of the actin cytoskeleton and decreased actin filament stability *In Vivo*. Direct association of Swf1p with actin is not predicted. The C-terminal region of Swf1p, modeled as cytoplasmic, did not bind G- or F-actin directly *in vitro*. Importantly, mutant analyses revealed that although the DHHC motif is functionally relevant to at least one growth-related Cdc42p function the actin regulating activity of Swf1p is independent of its DHHC motif. A *swf1* mutant, in which Ala substituted for the Cys required for the palmitoylation activity of DHHC-CRD pamitoyltransferases, displayed wild-type actin organization and Cdc42p localization. Bgl2p-marked exocytosis was found wild-type in this mutant, though invertase secretion was impaired. These data indicate Swf1p has at least two distinct functions, one of which regulates actin organization and Bgl2p-marked secretion. This report is the first to link the function of a DHHC-CRD protein to Cdc42p and the regulation of the actin cytoskeleton.

2523/B232

Insights into the Regulatory Mechanism of the Formin FHOD1.

A. Schönichen[1], O. Fackler[2], M. Geyer[1]; [1]Physical Biochemistry, Max-Planck-Institut for Molecular Physiology, Dortmund, Germany, [2]Institut für Virologie, Universität Heidelberg, Heidelberg, Germany

The actin cytoskeleton is essential for the shape of eukaryotic cells and cell motility and has as well a special role in muscle contraction. Formin proteins belong to the most important organizers of the actin filament formation. This group of proteins has a multidomain organization and a length of about 1000 aa. One common feature is the formin homology 2 domain (FH2) which is able to mediate actin nucleation at the barbed end of actin filaments. The diaphanous related formins (DRFs) contain a C-terminal autoregulatory domain, which binds to the N terminus of the molecule to inactivate this nucleation activity. Here we studied the N terminus of a human DRF, the formin homology domain 2 containing protein FHOD1. Structure determination of the N terminus of FHOD1 revealed two tightly linked domains: an armadillo repeat fold and which recognizes the diaphanous autoregulatory domain (DAD) and a GTPase binding domain with an ubiquitin like structure. Whereas the FH3 domain is similar to the FH3 domain of mDia, the GBD is very different from the mDia GBD which does not comprise a ubiquitin like structure (Schulte et al, 2008). It was shown recently that the C terminus of FHOD1 is phosphorylated by the Rho kinase ROCK1 (Takeya et al., 2008). By mutation of the C terminus we generated one construct mimicking a phosphorylated C terminus of FHOD1 and another one, which is inactive by replacing possible phosphorylation sites to alanines. In actin polymerization assays we could confirm the two activation states of these two mutants. Thus, phosphorylation of the C terminus is sufficient for the activation of FHOD1 in vitro. We could show in mammalian cell lines that FHOD1 could only be activated in presence of this GBD domain. These results suggest that not only the autoinhibitory interaction and its release by phosphorylation is important for the regulation but also the recruitment and orientation of FHOD1 within the cell is essential for the nucleation activity.

2524/B233

Contractile Ring Assembly in Fission Yeast: Parameter Dependence of Search, Capture, Pull and Release Model.

N. Ojkic, D. Vavylonis; Lehigh University, Bethlehem, PA

The contractile ring that forms during cytokinesis of fission yeast assembles from ~65 membrane-bound "nodes" which are macromolecular structures containing myosin-II Myo2p and formin Cdc12p. The nodes are initially distributed within a broad band at the cell center. Microscopic observations of stochastic node motions supported a "search, capture, pull and release" (SCPR) mechanism as the underlying process for the self-assembly of the contractile ring (Vavylonis et al. Science 97:319, 2008). In this model, actin filaments nucleated by formins at the nodes establish transient connections among them; the force generated by myosin motors on actin filaments pulls the nodes together into a ring. We used scaling arguments, analytical theory, and numerical simulations to explore the dependence of the results of the SCPR model on parameter values, in order to (i) examine the robustness of the proposed mechanism in assembling uniform rings, (ii) generate predictions for the results of experiments involving genetic modifications and drug treatments that correspond to changes in the model's parameter values, and (iii) examine how the SCPR model may relate to contractile actomyosin dynamics in systems beyond fission yeast. We found that uniform 2d distributions of nodes are stable over short times due to the randomness in the pattern of transient connections among nodes. Over sufficiently long times, uniform distributions collapse to disconnected node aggregates originating in random concentration fluctuations in the initial node distributions. Successful condensation of nodes into a ring requires that the lateral contraction of the broad band of nodes occurs much faster than clump formation. We calculated expressions that quantify the conditions for successful ring assembly and the ring assembly time as a function of the rate of establishment of connections, the spatial extent of node interactions, node concentration, the distance traveled per connection and the width of the band of nodes.

2525/B234

Zyxin Associates with Dynamic Actin Structures and Alters Cell Scattering Response in HGF Treated MDCK Cells.

R. Sperry, N. Bishop, S. Maxfield, Z. Lewis, D. Staley, R. Vellinga, M. Hansen; Physiology and Developmental Biology, Brigham Young University, Provo, UT

Epithelial cells adhere through cadherin-based junctions, which are coupled to the actin cytoskeleton by actin regulatory and binding proteins. Epithelial cells retain the ability to revert to a non-adherent state, dismantling adherens junctions (AJs) and becoming migratory, in a developmental process termed epithelial-mesenchymal transition, or EMT. EMT is an important process both during development and in pathologic states such as cancer metastasis. AJ breakdown requires local actin remodeling, but little is known about the identity or function of actin regulatory systems important to this process. MDCK cells in 2D culture undergo EMT in response to hepatocyte growth factor/scatter factor (HGF), providing a powerful model for addressing this problem. We use live cell imaging to analyze specific cellular events during MDCK cell scattering. HGF treated colonies respond by flattening, then contracting. Contraction coincides with junction rupture, forming expanding intracolony spaces. Points of persistent cell-cell adhesion result in formation of retraction fibers between separating migratory cells. Detailed quantitative analysis of these events enables comparison between parental MDCK cells and those with experimentally altered protein function. With this approach, we identify a role for the actin regulatory protein zyxin in cell scattering. MDCK cells expressing a mutant zyxin lacking its carboxy-terminal LIM domains exhibit slowed EMT response, characterized by a reduction in intracolony space formation and in number of retraction fibers. Retraction fibers that do occur persist much longer than in parental MDCK cells, increase to a greater length, and often fail to break. Immunofluorescence staining confirms an interaction between zyxin and actin reorganization during cell scattering, as zyxin colocalizes with dynamic actin structures at spots of persistent cell-cell adhesion. We propose zyxin regulates EMT by modifying dynamic actin reorganization during HGF induced cell scattering. Quantitative analysis of HGF induced MDCK cell scattering provides a powerful tool to identify novel regulators of cell scattering.

2526/B235

Molecular Requirements for Stretch-Dependent Actin Stress Fiber Accumulation of Zyxin.

L. M. Hoffman, C. C. Jensen, M. Yoshigi, M. Beckerle; Huntsman Cancer Institute, University of Utah, Salt Lake City, UT

Mechanical force causes the LIM-domain protein zyxin to mobilize from focal adhesions and accumulate along actin stress fibers, a process that is required for thickening and reinforcement of the actin cytoskeleton. Zyxin is not reported to be an actin binding protein but it interacts with proteins that bind actin. We hypothesized that zyxin recruitment to actin filaments is mediated through proteins that serve to bridge zyxin and actin. Candidates include Ena/VASP (E/V) family members, Cysteine-Rich Protein (CRP) family members, and α-actinin. If zyxin recruitment to stress fibers upon mechanical stimulation is dependent on one of these proteins, zyxin should fail to accumulate along actin in cells lacking these proteins, or when the partner binding sites on zyxin are compromised. We tested several genetically-null fibroblast cell lines and cells expressing mutant zyxin with application of uniaxial cyclic stretch followed by fixation, then visualized actin by phalloidin staining and zyxin with indirect immunofluorescence microscopy. In VASP-null cells, stretch-induced accumulation of zyxin occurred normally. Likewise, a mutant zyxin designed to disrupt interaction with all E/V proteins accumulated on actin following stretch, consistent with the notion that E/V proteins are not required for zyxin's recruitment or retention on actin after mechanical stimulation. Cyclic stretch of CRP1-null cells induced a normal zyxin localization along stress fibers, suggesting that the zyxin-actin interaction is not dependent on CRP1. Zyxin's N-terminus binds α-actinin, in addition to actin binding proteins LASP1 and LIM-nebulette. An N-terminal deletion of zyxin responded normally to mechanical stimulation, indicating zyxin interactions with α-actinin, LASP1 and LIM-nebulette are dispensable for zyxin relocalization in response to mechanical stress. These studies suggest a novel protein mediates zyxin's accumulation on stress fibers in response to cyclic stretch. Elucidation of zyxin recruitment to the actin cytoskeleton will enhance our understanding of how cells respond to mechanical cues.

2527/B236

Helical Twist Controls the Thickness of F-Actin Bundles.

C. Semmrich[1], M. M. Claessens[2], A. R. Bausch[1]; [1]Lehrstuhl fuer Zellbiophysik E27, Technische Universitaet Muenchen, Garching, Germany, [2]Mesa+ Institute for Nanotechnology and Biophysical Engineering Group, University of Twente, Enschede, Netherlands

Bundles of F-actin are key components for the mechanical support of eukaryotic cells. The appearance of the bundles is tightly controlled by a myriad of actin binding proteins (ABP). Moreover, each cytoskeletal process employs its own set of ABPs which results in a well defined length and thickness of bundles. Using an *in vitro* model system, we show that the maximal bundle size results from the mismatch between the helical structure of the individual filaments and the hexagonal packing within the bundle. Small angle x-ray scattering experiments reveal that the small and rigid ABP fascin changes the twist of the F-actin filament in a concentration dependent manner. This limits the maximal thickness to about 20 filaments per bundle. Additional crosslinking proteins can subsequently organize these small bundles into larger structures. The energetic tradeoff between filament twisting and crosslinker binding is suggested to be a fundamental mechanism by which cells can precisely adjust bundle size and strength.

2528/B237

Periodic Actomyosin Contractions in Xenopus Oocyte Extract.

G. A. Anderson[1,2], D. Strickland[1,3], T. Mitchison[1,4], C. Field[1,4]; [1]Physiology Course, Marine Biological Laboratory, Woods Hole, MA, [2]Department of Chemical and Systems Biology, Stanford University School of Medicine, Stanford, CA, [3]Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, IL, [4]Department of Systems Biology, Harvard Medical School, Boston, MA

Contractions occur in Cytochalasin D-free *Xenopus* oocyte extract upon heating to room temperature. These contractions are actin- and myosin-dependent, and they occur with regular period. We observed actomyosin contractions as flows of particulate extract in glass coverslip chambers. Actomyosin waves interact with old wavefronts, displaying a characteristic width of gelled material. We measured the period of the contractions and found that the addition of Cytochalasin D to extract decreases the period. We observed particulate flow in several distinct layers. Near the coverslips, flow occurs in the direction retrograde to contraction. This retrograde flow is a possible mechanism for recycling actin to nascent waves. Our results suggest a model of actomyosin contraction based on polymerization, gellation, and a contraction that carries with it F-actin above a critical length.

2529/B238

Temporal Aspects of Mechanosensing and Reponses.

R. Kemkemer, S. Jungbauer, A. Goldyn, J. P. Spatz; Max-Planck-Institute Stuttgart, Stuttgart, Germany

Shape and various functions of many cell types are controlled by mechanical forces and cells follow sequences of mechanosensing, transduction of the signal, and finally a mechanoresponse. The understanding of the dynamics and temporal characteristics of these processes is still poorly developed. We use an established technique of cyclically stretched substrate on which cells adhere. Using this setup, life cell observation during the mechanical manipulation revealed the dynamic of specific responses. Moreover we propose that the manipulation of the cells over a wide range of frequencies (temporal variation of forces) can serves as a new type of spectroscopy-like methods and is suitable to detect temporal characteristics of the sensing, transduction, and responses. In particular, we observed the dynamic reorientation of different fibroblast types over a frequency range 0.0001 to 20s-1 and strain amplitudes from 1% to 15%. We report quantitative measurements demonstrating a mean cell orientation with exponentially time characteristics. The characteristic time t is frequency-dependent and is in a range from 1 to 5 hours. At low cell densities, this characteristic time as a function of frequency has two characteristic regimes. For frequencies below 1s-1, t decreases with a power law as the frequency increases. For frequencies above 1s-1, t is nearly constant and the kinetics of cell reorientation is in saturation. In addition, a threshold frequency is found below which no significant cell reorientation occurs. Our results are consistent for the two different fibroblast types and indicate a saturation of molecular mechanisms of mechanotransduction or response machinery for sub confluent cells within the frequency regime under investigation. Further studies revealed the contribution of different cytoskeleton elements and focal adhesions to these observations.

2530/B239

Side-View Atomic Force Microscopy Reveals Three-Dimensional Cytoskeleton Reorganization in Response to a One-Dimensional Applied Stress.

O. Chaudhuri[1,2], S. Parekh[1,2], W. A. Lam[1,2], D. Fletcher[1,2]; [1]Bioengineering, University of California, Berkeley, Berkeley, CA, [2]UCSF/UC Berkeley Joint Graduate Group in Bioengineering, University of California, Berkeley, CA

In recent years, mechanical interactions between adherent cells and the extracellular matrix have been found to play an important role in a variety of biological processes including cell differentiation, proliferation, and tissue organization. Atomic force microscopy (AFM) has emerged as a powerful tool to quantify forces and mechanical properties at the cellular level. For example, the contractile force exerted by an adherent cell can be directly measured with AFM by attaching the cell between an AFM cantilever and a surface. However, it is difficult to correlate contractile force with changes in cell shape and cytoskeletal organization due to imaging limitations. Conventional AFMs can be combined with epi-fluorescence imaging systems that provide “bottom-view” images of a sample in a plane parallel to the surface. However, the most significant deformations of a cell attached between the cantilever and a surface will be in the direction of the applied force, which is in a plane perpendicular to the surface. To directly observe these deformations and associated cytoskeletal rearrangements, we have developed a “side-view” AFM that provides high magnification fluorescent images of the side profile of a sample during high-resolution force measurements. Using this side-view AFM, we studied the contractile response of individual U2OS human osteosarcoma cells, stably transfected with GFP-actin, to fixed and dynamic loading conditions while simultaneously imaging the actin cytoskeleton, cell membrane, and nucleus. We found that the cell changes its shape from rounded to “hourglass” shaped when it first adheres to the fibronectin coated cantilever and surface, and exerts increasing contractile forces on the cantilever. We observe the formation of actin stress fibers oriented both along and perpendicular to the loading axis, often linked together, as the cell continues contraction. Our results demonstrate a correlation between total force of contraction and dynamic organization of the actin cytoskeleton, pointing to an important link between the stress fibers anchoring adhesion sites and those that control cell shape.

2531/B240

Protein Unfolding/Remodeling in Tensed vs Relaxed Myotubes by Cys Shotgun Analyses.

C. Carag, D. Discher; University of Pennsylvania, Philadelphia, PA

Cytoskeletal proteins have evolved to resist the myriad of forces experienced by the cell in the course of its growth and development. It has been speculated that some cytoskeletal proteins have domains that unfold in response to force. Muscle cells in particular produce forces of large magnitude, but whether these forces are sufficient to cause protein unfolding or rearrangement is not known. In this study, a novel in-cell cysteine labeling techinique was used to detect possible protein conformation changes in intact, chemically relaxed or tensed skeletal myotubes. Like thiol labeling in solution, experimental conditions that lead to unfolding are shown by increased modification of hydrophobically labeled cysteines. 1D SDS-PAGE of cell lysates revealed five high molecular weight protein bands that have greater labeling when the myotube is tensed than relaxed. Mass spectrometry analysis, used to assess modification levels of individual proteins, indicated that cytoskeletal proteins such as filamin and talin undergo structural changes during muscle cell contraction.

2532/B241

Proteome-Level Unfolding and Changes in Assembly within Cells Probed by Cys Shotgun Kinetics in a Heat Shock Response.

B. Chase, J. Pajerowski, D. Discher; Bioengineering, University of Pennsylvania, Philadelphia, PA

Protein unfolding, disassembly, and aggregation underlie many diseases, but detailed study of these processes in intact cells has been limited. Cysteine Shotgun labeling utilizes cell-permeable fluorescent dyes to label exposed cysteine residues and was initially applied to study protein structure changes in response to mechanical stress on cells. We have re-purposed the technique to identify protein changes in whole-cell lysates in native versus urea-denaturing conditions and in live cells all as a function of time and temperature (20-45 deg-C). A protection factor is calculated for any given protein (and eventually Cys site) by normalizing the protein labeling kinetics to the rapid labeling under denaturing conditions. Proteins can be identified and further analyzed by mass spectrometry to pinpoint specific, susceptible domains involved. A number of proteins have been found to have protection factors that decrease dramatically in cells anywhere between 30-45 C. These proteins include structural proteins such as Filamin A and B, Talin 1, Myosin 9 and the Tubulin alpha-1B chain, heat shock protein 1 and pyruvate kinase isozymes. Some of these various proteins contain many cysteine-rich domains and thus appear especially amenable to studying by this new in-cell technique.

2533/B242

MYPT1 in Endothelium: Cloning and Functional Characterization.

K. Kim[1], C. Csortos[2], I. Czikora[2], N. Umapathy[1], G. Olah[2], I. Kolosova[3], D. Adyshev[4], A. Verin[1]; [1]Vascular Biology Center, Medical College of Georgia, Augusta, GA, [2]Molecular Medicine, University of Debrecen, Debrecen, Hungary, [3]Department of Medicine, Case Western Reserve University, Cleveland, OH, [4]Department of Medicine, University of Chicago, Chicago, IL

We and others have previously shown that myosin light chain (MLC) phosphatase (MLCP) is intimately involved in endothelial cell (EC) barrier regulation. However, the properties of MLCP in endothelium are poorly understood. Our data demonstrated that specific depletion of MLCP targeting subunit (MYPT1) by siRNA technique significantly attenuated EC barrier enhancement, induced by extracellular ATP. Vice versa, ectopic expression of constitutively-active (c/a) MYPT1 fragment (MYPT1 1-300) in human EC inhibited the phosphorylation of MLC, F-Actin formation, and the decrease in transendothelial electrical resistance (TER) induced by edemagenic agent, thrombin. Consistent with these data, the increase in TER induced by EC barrier-protective agent (sphingosine-1-phosphate) was enhanced. In order to further characterize MYPT1 properties in EC, we cloned this protein in human and bovine EC. Using RT-PCR and library screening we identified 2 isoforms which differ by the presence (Long) or absence (V2) of a central 168bp insert (between 1657-1824 bp for human) with previously unknown function. To examine the functional differences between these 2 variants we co-expressed them with cytoskeletal proteins of ezrin/radixin/moezin (ERM) family, novel substrates of MLCP, in COS7 cells. In co-imunoprecipitation experiments, we found that radixin preferentially binds with MYPT1 Long compare to MYPT1 V2 although both of MYPT1 isoforms have radixin binding sites. Co-expression experiments of ectopically expressed ERM proteins with either MLCP catalytic subunit (CS1β) or MYPT1 constructs revealed that ezrin and moesin preferentially bind with CS1β rather MYPT1. These results indicated that interaction of MLCP with ERM proteins is complex and suggest the possibility that MYPT1 isoforms may have distinct role in alignment of the cytoskeleton through ERM-driven membrane dynamics.

2534/B243

The Dynamics of Interaction of S100A4 with Myosin IIA.

C. R. Bagshaw[1], S. K. Badyal[1,2], I. L. Barsukov[3], J. Basran[1], A. F. Irvine[1,2], M. Kriajevska[2]; [1]Biochemistry, University of Leicester, Leicester, United Kingdom, [2]Cancer Studies and Molecular Medicine, University of Leicester, Leicester, United Kingdom, [3]Biological Sciences, University of Liverpool, Liverpool, United Kingdom

S100A4 is a Ca2+ binding protein associated with the modulation of various cellular processes including motility and migration. Up-regulation of S100A4 was observed in various carcinoma cell lines undergoing an epithelial-mesenchymal transition (EMT). We are investigating the interaction of S100A4 with one of its targets, non-muscle myosin IIA, both in vitro and in A431 cells. Ca2+ binding to S100A4 has a Kd of 5 μM and a dissociation rate constant of 20 s-1 as monitored by intrinsic tyrosine fluorescence and competition with Quin-2. Both Y19 and Y75 contribute to the tyrosine signal. Addition of a 111 residue C-terminal myosin IIA fragment reduces the rate constant of Ca2+ dissociation to 0.6 s-1, and is likely to be limited by myosin dissociation from the ternary complex. Induction of SIP1 in A431 cells induces EMT and increased S100A4 levels. Such cells show limited ability to form stress fibers, as imaged by the expression of GFP-myosin IIA. Reduction of S100A4 levels by siRNA is associated with more extensive stress fibers. Fluorescence recovery after photobleaching (FRAP) studies indicated the exchange between cytoplasmic and polymerised myosin was faster in the presence of S100A4. However these data do not necessarily indicate a direct effect of S100A4 on the disassembly of myosin filaments. The binding of S100A4 to monomeric myosin would reduce the extent of myosin filament formation and could speed up the kinetics of exchange by providing a

buffered pool of cytoplasmic myosin. Without such a pool, most of the myosin is present within polymerised structures and FRAP requires monomeric myosin to diffuse a greater distance in order to replenish the bleached GFP-myosin.

2535/B244

Regulation of Microglia Chemotaxis by VASP Phosphorylation.

S. Lee, C. Chung; Vanderbilt University Medical Center, Nashville, TN

Microglia are immune effector cells in the brain and participate in the response to brain injuries and diseases in which their activation, migration, and proliferation are known to play crucial roles. It was previously reported that extracellular ADP could induce microglia chemotaxis and membrane ruffle formation through P2Y12 receptor. However, the intracellular signals underlying microglia migration is poorly understood. In this study, we examined the role of VASP phosphorylation in ADP-induced microglia chemotaxis and membrane ruffle formation. ADP stimulation transiently increased intracellular cAMP concentration, VASP phosphorylation at Ser157, membrane ruffle formation, and chemotaxis in microglia. P2Y12 receptor antagonist and PKA inhibitor effectively inhibited VASP phosphorylation and chemotaxis. These results indicate that P2Y12-mediated activation of PKA and subsequent VASP phosphorylation are involved for the regulation of microglia chemotaxis in response to ADP. Forskolin (FSK), an adenyl cyclase activator, induced VASP phosphorylation sustained longer at high level. The retraction of membrane ruffles in FSK- or okadaic acid (OA)-treated cells was significantly reduced, leading to reduced chemotaxis. In FSK- or OA-treated cells, phosphorylated VASP remained at the membrane cortex and size and number of focal adhesions were not increased compared to increased number and size of focal adhesions in cells stimulated with ADP. This result indicates that prolonged phosphorylation of VASP could inhibit transformation of focal complexes into focal adhesions. Our results suggest that VASP phophorylation through P2Y12 receptor and PKA activation plays an important role in membrane ruffle formation and chemotaxis via the regulation of focal adhesion formation/maturation.

2536/B245

Mechanics of Epithelial Morphogenesis in Drosophila Melanogaster Oogenesis.

K. S. Kolahi[1,2], A. Clasen[2], P. F. White[1], D. Shreter[1], D. Bilder[2], M. Mofrad[1]; [1]Bioengineering, University of California, Berkeley, Berkeley, CA, [2]Molecular and Cell Biology, University of California, Berkeley, Berkeley, CA

Posterior migration is a conserved developmental process in epithelial tissues of various organisms. In the Drosophila melanogaster egg chamber, posterior migration of the follicle epithelium takes place and simultaneously segregates the initial simple cuboidal epithelium into distinct squamous and columnar epithelia. The forces involved in the migration and the shape changes of follicle epithelial cells are poorly understood. In this study, we measure the morphological characteristics, surface areas, volumes, and positions of the cells and tissues to shed light on this process. Our data indicate that posterior migration in the Drosophila egg chamber is a misnomer, and implies active migration of the follicle cells. Instead, we propose that the migration is a perceived effect to the oocyte's rapid growth during the stages of posterior migration. In addition, we implicate the oocyte's rapid growth to imparting tensile stresses in the follicle epithelium, and thus cell shape can be a function cellular integrity.

2537/B246

Anisotropic Rheology and Directional Mechanotransduction in Vascular Endothelial Cells.

J. C. del Alamo[1], G. N. Norwich[2], J. Li[2], J. C. Lasheras[1,2], S. Chien[2,3]; [1]Mechanical and Aerospace Engineering, University of California San Diego, La Jolla, CA, [2]Bioengineering, University of California San Diego, La Jolla, CA, [3]Medicine, University of California San Diego, La Jolla, CA

Adherent cells undergo remodeling in response to external mechanical stimuli, with a consequent redistribution of intracellular forces. This redistribution depends on the composition and structural directionality of the cytoskeleton, and has been shown to play an important role in cell function. We analyzed the temporal and spatial changes in magnitude and directionality of the cytoplasmic shear modulus (G) in confluent cultures of bovine aortic endothelial cells subjected to continuous laminar flow shear stresses. For this purpose, we used directional particle tracking microrheology to analyze the Brownian dynamics of intracellular markers. Our approach considers the complete second-order tensor of marker displacements, allowing us to determine at each position the principal directions of highest and lowest G. After the application of shear, the random motion of the mitochondria is tracked every 30 minutes using phase-contrast microscopy. This non-invasive method has been validated by tracking micro-beads injected into static cells. Under static condition, the cells have polygonal shapes without specific alignment. Although G of each cell exhibits directionality with varying principal directions, it is isotropic when averaged over the whole cell population. After continuous laminar shear stresses, the cells elongate and the stress fibers gradually reorient parallel to the flow. Our measurements show that, as the stress fibers become aligned with the flow, the local regions with the lowest G are oriented parallel to the flow, while those with the highest G are oriented perpendicular to it. This mechanical alignment is accompanied by a transition of the cytoplasm to be more fluid-like along the flow direction and more solid-like along the perpendicular direction; G also decreases at downstream part of the cells. The shear-induced directional anisotropy and spatial inhomogeneity of cytoplasmic rheology may play an important role in mechanotransduction in adherent cells by providing a means to sense the direction of mechanical stimuli. This work was supported by NIH grants 1R01 HL080518 and BRP HL064382. JCdÁ has been partially supported by the Spanish MEC (Fulbright Program).

Intermediate Filaments (2538 – 2545)

2538/B247

Variation of the NF-M KSP Repeat Sub-Domain among Mammalian Species: Implications for Altering Axonal Structure.

D. M. Barry, M. Garcia; Biological Sciences, University of Missouri, Columbia, MO

As larger mammals evolved and neurons grew longer, relatively fast nerve conduction velocities are likely to have been maintained to ensure survival. A plausible mechanism to explain the maintenance of rapid conduction velocity is a corresponding increase in axonal diameter with

evolving length. The tail domain of the neurofilament medium (NF-M) subunit is a determinant of axonal diameter in large caliber myelinated axons. Sequence analysis results of mammalian orthologs indicates that the NF-M tail domain contains a variable lysine-serine-proline (KSP) repeat sub-domain flanked by two highly conserved sub-domains. Moreover, the number of KSPs within the NF-M KSP repeat motif varies among species, yet does not change within species. Variations within the KSP repeat motif may have resulted in increased axon caliber, conserving nerve conduction velocity as mammals became larger.

2539/B248

The Biomechanical Properties of Intermediate Filaments: Implications for Epidermolysis Bullosa Simplex.

D. R. Beriault, D. Fudge; Integrative Biology, University of Guelph, Guelph, ON, Canada

Epidermolysis bullosa simplex (EBS) is a skin fragility disorder characterized by the separation and cytolysis of basal epidermal keratinocytes away from the dermis layer upon mild mechanical trauma. Dowling-Meara (DM) is the most severe form of EBS, and 70% of all EBS-DM cases are caused by an R125 dominant negative mutation in keratin 14 intermediate filament (IF) proteins (Russell et al., 2004). Recent studies have demonstrated that single IFs in vitro (Kreplak et al., 2005) and networks of IFs in cultured cells (Fudge et al., 2008) are remarkably extensible. We are investigating whether the recently elucidated tensile properties of IFs in vitro are relevant to the pathophysiology of EBS skin fragility in vivo. Current results comparing cells with WT and R125P IFs indicate a significant difference in cell viability during acute uniaxial cell stretches of 40% and 120%; however, no significant difference in viability was noted in 0%, 66% and 133% strains. Furthermore, GFP tagged R125P IFs in vivo appear to withstand acute stretches up to 133% without visible damage to the network. Remarkably, the mutant keratin aggregates found in the cell periphery before stretch appear to reassemble into the IF network during stretch. The IFs ability to resist these significant cell strains does not appear to require either the F-actin or tubulin networks. These results suggest that the mechanical fragility of R125P cells may be due to their more dynamic IF networks rather than a mechanical weakness of the IFs themselves.

2540/B249

Cytokine-Mediated Recruitment of Langerhans Cells to Lesional Epidermis in K5 -/- Mice and in Human Epidermolysis Bullosa Simplex.

W. Roth[1], T. Magin[1], U. Reuter[1], C. Wohlenberg[1], L. Bruckner-Tuderman[2]; [1]Biochemistry and Molecular Biology, Univ. Bonn, Bonn, Germany, [2]Department of Dermatology, University Medical Center Freiburg, Univ. Freiburg, Freiburg, Germany

Epidermolysis bullosa simplex (EBS) is a rare skin disorder caused by mutations in the keratin genes KRT5 and KRT14, expressed in the basal epidermis. EBS is widely regarded as a mechanobullous disease, resulting from an altered keratin cytoskeleton, causing extensive cytolysis and cell fragility of basal keratinocytes. There is a growing body of evidence for additional regulatory functions of keratins in epithelial tissues, like their function in translation control and organelle transport. Here, we demonstrate for the first time that the cytokines MCP-1/CCL2, MIP-3beta/CCL19 and MIP-3alpha/CCL20, involved in the recruitment, maturation, and migration of Langerhans cells in the epidermis are up-regulated in the skin of K5-/-, but not of K14-/- mice. MIP-1gamma/CCL9 was upregulated in both mouse models. In neonatal K5-/- epidermis, Langerhans cell numbers were increased 2-fold. Furthermore, enhanced Langerhans cell recruitment within the epidermis was found in five EBS patients with mutations in the KRT5 gene. In agreement with the NF-κB-dependent regulation of these cytokines, we found a decrease in p120-catenin in the basal epidermis of K5-/- mice. These data provide the first evidence that K5-mediated upregulation of distinct cytokines through p120-catenin may contribute to the pathogenesis of EBS.

2541/B250

Cytokeratins 8 and 18 in Normal Human Corneal Endothelium.

S. Merjavá[1], A. Neuwirth[1], K. Jirsova[1,2]; [1]Laboratory ot the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic, [2]Ocular Tissue Bank, General Teaching Hospital and First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

Objectives: To determine if cytokeratins (CK) 8 and 18 are expressed in healthy human corneal endothelium. Methods: Eleven human corneal discs not suitable for transplantation were used. The mean age of the corneal donors was 65 ± 12.7 years. Cryosections (7µm), paraffin-embedded sections and corneal endothelium imprints on Millicell membranes were used. Specimens were fixed in three different solutions and indirect immunofluorescent or enzymatic staining was performed using antibodies against CK8 (Chemicon), CK18 (Dako), CK18 (Sigma) and CK8/18 (Novocastra). Semi-quantitative RT PCR and Western blotting (cell RNA or proteins were isolated from Millicell membranes) were performed. The intensity of the staining was assessed using fluorescent microscopy, and the percentage of positive cells was calculated from at least two hundred cells. Results: The endothelium of all the healthy corneas formed a monolayer of flat cells with the hexagonal morphology typical for the corneal endothelium. Approximately 50 % of the cells stained positively for CK8 (Chemicon), CK18 (Sigma) and the CK pair 8/18 (Novocastra) in the corneal endothelium when acetone was used as a fixative. Four and 52 % CK18-positive cells were observed using immunofluorescent and enzymatic immunohistochemistry, respectively, when the CK18 antibody from Dako was used. No signal was detected using any of the tested antibodies when 4% formalin or 10% paraformaldehyde was used for fixation. Using semi-quantitative RT-PCR, CK8 and CK18 mRNAs were detected in the endothelium of all tested corneas. Both cytokeratins were detected in the endothelium of all tested corneas by Western blotting as well. Conclusion: We have detected both CK8 and CK18 in the endothelium of all specimens at both the protein and mRNA levels. These results clearly demonstrate that cells of the corneal endothelium express CKs 8 and 18 and share some features with simple epithelia.

2542/B251

Keratins Modulate Colonic Mitochondrial HMG-Coa Synthase and Ketone Body Metabolism.

L. Fortelius[1], A. Habtezion[2], J. Liao[4], S. Azhar[2], M. Omary[3], D. Toivola[1,2]; [1]Biology/Biosciences, Åbo Akademi University, Turku, Finland, [2]VA Palo Alto Health Care System and Stanford University Digestive Disease Center, Palo Alto, CA, [3]Univerisity of Michigan, Ann Arbor, MI, [4]Applied Biomics, Hayward, CA

Keratin 8 (K8) is the major type II intermediate filament protein (IF) of the intestinal epithelium. K8 was the first IF protein targeted for deletion in transgenic mice. In addition to a profound liver injury susceptibility phenotype, K8-null mice develop within 2-3 weeks of birth, colonic hyperproliferation and Th-2-type colitis that resolves after antibiotic therapy. K8-null enterocytes also mistarget membrane proteins (e.g. ion transporters) and are less susceptible to apoptosis compared to wild type (WT) cells. A proteomics approach was used to probe keratin function in the colon, and the colitis that is induced by keratin absence. Proteins were isolated from WT and K8-null colonic crypts and labeled with Cy3 (WT) and Cy5 (K8-null). The combined samples were then analyzed using two-dimensional differential in gel electrophoresis (2-D DIGE) coupled with mass spectrometry. In K8-null colonic crypts, the major down-regulated protein was the mitochondrial 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMGCS2), which is the rate-limiting enzyme in ketogenesis producing ketone bodies for colonocyte fuel, and is important during starvation. The four major HMGCS2 charged isoforms were downregulated 3-4 fold in K8-null colonic crypts. The decreased expression of HMGCS2 was confirmed at the protein (by immunoblotting and immunofluorescence staining) and RNA levels. Changes in HMGCS2 were specific to the colon and were not found in the liver or pancreas. Peroxisome proliferator-activated receptor α (PPARα), an HMGCS2 transcriptional activator, was also down-regulated in K8-null colon. Moreover, induction of ketogenic conditions by 24-hr starvation or 3-5 day ketogenic diet increased HMGCS2 in WT mice as expected, but this response was blunted in K8 null colonocytes. K8-null male mice, but not K8-null female or WT mice, lost weight upon ketogenic diet feeding. Keratin phosphorylation was not significantly affected by starvation, thereby suggesting that keratin phosphorylation is not essential in this regulation. These results suggest that colonic keratin IFs are involved in colonocyte ketone body and energy metabolism.

2543/B252

Rescue of Atypical PKC: Role of the Hsp70 Chaperone and Intermediate Filaments in Polarized Epithelial Cells.

A. Mashukova, A. S. Oriolo, F. Wald, P. J. Salas; Cell Biology, Univ. of Miami School of Medicine, Miami, FL

Interaction of conventional PKC isoforms with Hsp70 was shown to sustain the signaling life time of the kinase by protecting it from activation induced dephosphorylation and degradation. In this work, we sought to assess if atypical PKC (aPKC, PKCι) undergoes the same conformational rescue cycle mediated by Hsp70. The rationale for this hypothesis is that the same invariant Leu domain that is responsible for the Hsp70 - PKCβ interaction is fully conserved in PKCι. We performed confocal colocalization experiments, differential extraction and immunoblot analysis, qPCR and shRNA-mediated knock-down of Hsp70 and keratins in Caco-2 (human colon carcinoma) epithelial cells. Also, we designed an in vitro reconstitution assay using different fractions of the same cells to better understand the compartmentalization of the key players in the PKCι rescue process. In this assay we obtained soluble, actin and keratin fractions, and used them separately or in pairs to induce rephosphorylation of the PKCι turn domain. The results indicate that both Hsp70 and PKCι exist in two different pools: soluble and associated to the cytoskeleton. Interestingly, a small pool of enzymatically active PKCι can associate to intermediate filaments in the absence of its most common partners, Par3 and Par6. Reconstitution assays and shRNA knock-downs show that both Hsp70 and the native filamentous keratin cytoskeleton are necessary for the rephosphorylation of the inactive form of PKCι and its rescue from degradation. In the absence of detectable changes of PKCι mRNA, we conclude that this is a post-translational mechanism essential to maintain physiological steady-state levels of PKCι, and that it may also contribute to its normal polarization.

2544/B253

Role of Akt Isoforms in Regulating Expression of Intermediate Filament Proteins, Keratin 8/18 and Vimentin, in Cancer Progression.

A. Fortier, É. Asselin, M. Cadrin; Chimie-Biologie, University of Quebec at Trois-Rivières, Trois-Rivières, QC, Canada

Keratins are epithelial-specific intermediate filament (IF) proteins, which are expressed in a tissue-specific manner. As part of the cytoskeleton, keratins are important for the mechanical stability and integrity of epithelial cells and tissues. Moreover, some keratins are involved in intracellular signalling pathways which regulate cell response to injuries and nonmechanical stresses, cell growth, cell death and cancer progression. Keratins 8 and 18 (K8/18) are expressed in most carcinoma cells. However, in late-stage cancer, vimentin is often co-expressed with keratins in cells presenting a dedifferentiated phenotype. This is associated with an increased metastatic potential. Metastasis, the foremost cause of mortality in cancer patients, is a coordinated biological process involving multiple intracellular signalling pathways. PI3K/Akt pathway has recently been shown to play a role in invasion/metastasis, and the three Akt isoforms would play differential roles in this process. Our laboratory investigates the role of Akt isoforms in regulating the expression of keratins and vimentin in different cancer cell lines, and the possible implication of IFs in Akt-induced cell invasion and metastatic potential. In the present study we used endometrial, cervical and breast cancer cell lines and analysed the effect of Akt2 over expression and activation or inhibition on K8, K18 and vimentin levels. The metastatic potential of the cells was also analysed. Our results show that overexpression of constitutively active Akt2 isoform increases K18 and vimentin expression while K8 expression is decrease. These phenomena are associated with an increase in cellular invasiveness. The inhibition of Akt activity using LY294002 induces a decrease in IFs levels. These results represent, to our knowledge, the first direct evidence that Akt activity is regulating IFs expression. More over, our results support the hypothesis that modulation of IFs expression plays an active role in cancer progression. This work was supported by an NSERC grant to M.Cadrin.

2545/B254

Characterization of Rod 1A and Identification of Head-Rod Interaction Sites of Vimentin.

A. Aziz[1], J. F. Hess[1], M. S. Budamagunta[2], J. C. Voss[2], P. FitzGerald[1]; [1]Cell Biology and Human Anatomy, School of Medicine, University of California, Davis, CA, [2]Biochemistry and Molecular Medicine, School of Medicine, University of California, Davis, CA

Purpose: To test the longstanding predictions that rod 1A of vimentin is assembled into a coiled-coil dimer in intact IFs and hypothesis that head domain folds back on the rod using SDSL-EPR approach. Materials and Methods: Cysteines were placed in vimentin at sites of interest using basic genetic engineering approaches. The mutants were expressed in bacteria, purified by FPLC, and spin-labeled using O-87500 [MTSL-d15]. Labeled proteins were assembled into filaments by dialysis from 8M urea and filament assembly was checked by electron microscopy. EPR measurements were carried out in JEOL X-band spectrometer fitted with a loop-gap resonator. Results: The SDSL EPR spectral data indicates that the rod 1A from residue 120-141 has α-helical coiled coil conformation. The d1/d score (dipolar broadening ratio)

collected from frozen samples at -100°C showed the periodicity of the distance separation along the positions of the heptad, in what constitutes a "coiled coil EPR signature". The heptad repeat pattern is interrupted after position 142, suggesting some kind of transition out of the α-helix coiled coil pattern into non α-helical coiled coil. To search for head-rod interactions, multiple mutants bearing spin labels in the head were mixed with mutants in the rod 1A region. We found that the spin label at position 17 in the head region showed maximum proximity to the spin label at residue 137, a "c" position in the Rod 1A as compared to spin labels placed up- and downstream of each site. To explore the progression of 17-137 interactions during in vitro assembly, spectra of samples (17-17, 137-137 and 17-137) undergoing stepwise assembly were monitored. The results suggest that the specific association between head and rod domains occurs at an early stage in assembly process. Conclusions: The data points to coiled coil structure within Rod 1A from position 120-141, with a deviation from coiled-coil structure occurring within the region 142-145 which points towards the start of Linker L1. The data also provide evidence that head folds back onto Rod 1A and comes into close proximity for the interaction between 17 (head) and 137 (Rod 1A) to occur and that this interaction occurs very early in the assembly formation.

Cilia and Flagella IV (2546 – 2568)

2546/B255

A Rab11/Rab8 Switch Regulates Membrane Transport to Nascent Primary Cilia.

C. J. Westlake[1], R. H. Scheller[1], P. K. Jackson[1], M. V. Nachury[2]; [1]Genentech, South San Francisco, CA, [2]Molecular and Cellular Physiology, Stanford University Medical School, Palo Alto, CA

Bardet-Biedl syndrome (BBS) is a ciliopathy whose symptoms include obesity, retinal degeneration, and kidney cysts. We have recently shown that the nucleotide exchange factor (GEF) for Rab8, Rabin8, interacts with a core complex of BBS proteins (the BBSome) and that activation of Rab8 is required for ciliogenesis. However, how Rab8 directs ciliary growth remains an open question. Through real-time imaging of RPE cells stably expressing GFP-Rab8, we found that Rab8 localizes to nascent cilia at the onset of cilia formation. Surprisingly, influx of Rab8 into cilia ceases following organelle maturation and Rab8 is progressively lost from mature cilia. Interestingly, Rab8-positive membrane tubules are detected at the base of the cilium. To gain insight into the regulation of Rab8, we performed a directed yeast 2-hybrid screen of Rabin8 against a comprehensive collection of Rabs. Strikingly, we discovered a specific interaction between $Rab11^{GTP}$ and Rabin8. Co-immunoprecipitation studies confirmed that Rabin8 indeed associates with Rab11. This interaction was not to the Sec2/GEF domain, but to a region known to be important for membrane association. Consistent with this result, Rabin8 was detected on Rab11 vesicles near the base of the cilium. RNAi and dominant-negative overexpression studies demonstrated that Rab11 is required for ciliation. Finally, Rab11 vesicles are frequently associated with Rab8 tubulovesicular structures, and these structures failed to form in the absence of $Rab11^{GTP}$. We propose a model whereby Rab8 membrane transport to the cilia is stimulated by Rab11-targeted delivery of Rabin8 to the cilia base. In summary we have identified a novel function for Rab11 in regulating Rab8 tubulovesicular transport.

2547/B256

Targeting of β-Arrestin2 to the Centrosome and Primary Cilium: Role in Cell Proliferation Control.

A. Molla-Herman[1], C. Boularan[1], R. Ghossoub[1], M. G. Scott[1], A. Burtey[1], M. Zarka[1], S. Saunier[2], J. P. Concordet[3], S. Marullo[1], A. Benmerah[1]; [1]Infectious Diseases, Institut Cochin, Paris, France, [2]Pediatric Nephorlogy, Hôpital Necker Enfants Malades, Paris, France, [3]Genetique et Developpement, Institut Cochin, Paris, France

The primary cilium (PC) is a sensory organelle generated from the centrosome in quiescent cells and found at the surface of most cell types, from where it controls important physiological processes. Specific sets of membrane proteins involved in sensing the extracellular milieu are concentrated within cilia, including G protein coupled receptors (GPCRs). Most GPCRs are regulated by beta-arrestins, βarr1 and βarr2, which control both their signalling and endocytosis, suggesting that beta-arrs may also function at PC. In cycling cells, βarr2 was observed at the centrosome, at the proximal region of the centrioles, in a microtubule independent manner. However, βarr2 did not appear to be involved in classical centrosome-associated functions. In quiescent cells, both in vitro and in vivo, βarr2 was found at the basal body and axoneme of PC. Interestingly, βarr2 was found to interact and colocalize with 14-3-3 proteins and Kif3A, two proteins known to be involved in ciliogenesis and intraciliary transport. In addition, as suggested for other centrosome or cilia-associated proteins, βarrs appear to control cell cycle progression. Indeed, cells lacking βarr2 were unable to properly respond to serum starvation and formed less PC in these conditions. Our results show that βarr2 is localized to the centrosome in cycling cells and to the PC in quiescent cells, a feature shared with other proteins known to be involved in ciliogenesis or PC function. Within cilia, βarr2 may participate in the signaling of cilia-associated GPCRs and, therefore, in the sensory functions of this cell "antenna".

2548/B257

The Primary Cilium Coordinates Hedgehog Signaling in the Mouse P19.CL6 Embryonal Carcinoma Stem Cells.

C. A. Clement[1], S. G. Kristensen[1], L. A. Larsen[2], S. T. Christensen[1]; [1]Department of Cell and Developmental Biology, Institute of Biology, Copenhagen, Denmark, [2]Wilhelm Johannsen Centre for Functional Genome Research, University of Copenhagen, Copenhagen, Denmark

The pluripotent P19.CL6 cell is an isolated sub clone from the murine embryonal carcinoma (EC) P19 cell line and is an exceptional model to study early cardiac differentiation because the cells can differentiate into beating cardiomyocytes *in vitro*(Skerjanc,1999,TCM139-143). The important transcription factors during the early cardiac development are the members of the Gata family and the homeobox protein family Nkx2-5 (Sucov,1998,Annu Rev Physiol 287-308). Furthermore, the early cardiac development is controlled and induced by Hedgehog (Hh) signaling in P19 cells (Gianakopoulos et al,2005,JBC21022). The essential Hh signal components have been shown to localize to primary cilia in for example human embryonic stem cells, indicating that the Hh pathway is regulated though this organelle in development (Christensen and Ott,2007,Science317-30; Enko et al, 2008,JCB 897-904). In this study we investigated the role of primary cilia in P19.CL6 cells during early cardiac development. Cyclopamine, a specific Smoothened (Smo) inhibitor (Chen et al,2002,Genes Dev2743-48) has severe negative effect on Hh signaling and cardiac development. Cyclopamine treated cells fail to express proper mRNA and protein levels of

the transcription factor Gata4 and Nkx2-5 and fail to develop cardiomyocytes. Furthermore, cyclopamine treatment leads to altered Gli2 processing shown with western blotting and altered Gli3 translocation visualized by immunofluorescence microscopy (IF). Gli1 is highly up-regulated in controls compared to cyclopamine treated cells on both mRNA levels as judged by Q-RT-PCR analysis and on the protein level as judged by IF analysis. In general cyclopamine shuts down Hh-signaling in P19.CL6 cells and results in cell cultures with no beating cardiomyocytes. The Hh components Gli2, Smo and Patched localize to primary cilia and leads to our hypothesis that the primary cilium controls differentiation of cells into cardiomyocytes and partly via ciliary Hh signaling. Supporting this hypothesis we have also shown that knockdown of IFT88 and IFT20 by nucleofection results in very few beating cardiomyocytes and a reduced mRNA level of various Hh genes, including Patched-1 (Kristensen et al, please see accompanying poster).

2549/B258

The Primary Cilium Coordinates Early Cardiac Development and Differentiation of Mouse Embryonal Carcinoma Stem Cells into Cardiomyocytes via Hedgehog Signaling.

S. G. Kristensen[1], C. A. Clement[1], K. Mollgaard[2], B. K. Yoder[3], G. Pazour[4], L. A. Larsen[5], S. T. Christensen[1]; [1]Cell and Developmental Biology, University of Copenhagen, Copenhagen OE, Denmark, [2]Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen, Denmark, [3]Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL, [4]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, [5]Wilhelm Johannsen Centre for Functional Genome Research, University of Copenhagen, Copenhagen, Denmark

The P19 embryonal carcinoma cell line is an exceptional model to study early cardiac differentiation because the cells have the ability to differentiate into cardiomyocytes *in vitro* (Skerjanc,1999,TCM139-43). The first stage of cardiomyogenesis involves the formation of cardiomyoblasts, which express a distinct subset of transcription factors including members of the Gata family and the homeobox protein family Nkx2-5 (Sucov,1998,Annu Rev Physiol 287-308). Previous studies have indicated that cardiomyogenesis is coordinated by Hedgehog (Hh) signaling in P19 cells (Gianakopoulos et al,2005,JBC21022). We have investigated the role of the primary cilium in early cardiac development partly by IFT20 and IFT88/*Tg737*/Polaris plasmid siRNA in P19 cells and partly by immunohistochemical analysis of *Tg737*^{orpk} mice. IFT20 is important for transport of membrane proteins from Golgi to the cilia, and IFT88/*Tg737*/Polaris is essential for ciliogenesis, which when knocked down blocks ciliary assembly (Follit et al,2006, Mol Biol Cell3781-92; Pazour et al., 2000, J Cell Biol 709-18.). We find that siRNA of IFT88 and IFT20 by nucleofection blocks ciliary assembly in P19 cells, and this is accompanied by a reduction in the expression of Gata4 and Nkx2-5 during early cardiac development, which fail to form beating cardiomyocytes. Further, RNAi of the primary cilium is associated with a reduction in the transcription level of Hh genes, including Patched-1. *Tg737*^{orpk} mice have a loss of IFT88/Polaris caused by a hypomorphic mutation, which results in very few and short primary cilia (McGlashan et al,2007,Matrix Biol 234-46). By immunohistochemical analysis we show that homo- and heterozygous *Tg737*^{orpk} mice (e11.5) have heart developmental defects such as malformation of the atrial heart chambers compared to wild type mice. We therefore suggest that the primary cilium is necessary for differentiation of cells into cardiomyocytes, partly via ciliary Hh signaling. Supporting this hypothesis, we have also shown that primary cilia of P19 cells contain Hh signaling components such as Patched-1, which when inhibited by cyclopamine blocks the differentiation of P19 cells into cardiomyocytes (Clement et al, please see accompanying poster)

2550/B259

Tctex-1 Participates in the Non-Canonical Gβ Signaling Pathway for Neural Progenitor Fate Specification during Corticogenesis.

A. Li[1], J. Chuang[1], C. Dedesma[1], T. Yeh[1], K. Tomizawa[3], C. Sung[1,2]; [1]Margaret M. Dyson Vision Research Institute, Weill Cornell Medical College, New York, NY, [2]Cell and Developmental Biology, Weill Cornell Medical College, New York, NY, [3]Physiology, Okayama University Graduate School of Medicine, Okayama, Japan

Tctex-1, a cytoplasmic dynein light chain, also serves as an activator of G protein signaling (AGS2) that directly binds to Gβ subunit of heterotrimeric G proteins (Sachdev et al., 2007, EMBO J 26:2621). In light of the previous report suggesting that Gβ signaling and the GoLoco protein AGS3 (activator of G protein signaling 3) were involved in the cell fate specification of neural progenitors during neocortical development (Sanada and Tsai, 2005, Cell 122:119), we investigated the physiological relevance between Tctex-1 and G protein signaling in mouse neocortex. Using the in utero electroporation method, we showed that neural progenitors tend to exit cell cycle and differentiate into neuron upon Tctex-1 silencing, Gβγ signaling perturbation, or AGS3 silencing. Rescue experiments suggested that the phospho-mimic form Tctex-1 (that does not associate with the dynein complex) was able to rescue these phenotypes. Conversely, overexpressing phospho-mimic form Tctex-1 promoted cell proliferation by accelerating cell cycle entry. Antibodies specifically recognizing phospho-Tctex-1 showed it is concentrated near the basal bodies of primary cilia of neural progenitors. Cilia assembly/disassembly experiments carried out in cultured fibroblasts further suggested that phospho-Tctex-1 plays an important role in ciliary dynamics and its recruitment to the ciliary base depends on the non-canonical Gβ signaling pathway. These results collectively support a model in which Tctex-1 acts as a downstream effector of non-canonical Gβγ pathway and involved in cilium-mediated cell cycle progression and fate determination during mammalian corticogenesis.

2551/B260

The Cytoplasmic Tail of Fibrocystin Contains a Ciliary Targeting Sequence Which Interacts with the Small G Protein Arf4.

J. Follit, L. Li, G. Pazour; Molecular Medicine, University of Massachusetts Medical School, Worcester, MA

Primary cilia are found on nearly every vertebrate cell type and play vital roles in the development of mammals and in the etiology of diseases such as polycystic kidney disease and blindness. It is thought that the major function of these organelles is to perceive cues from the extracellular environment and communicate them to the cell body. The sensory functions of primary cilia rely on receptors and other membrane proteins localized to the ciliary membrane but little is known about how proteins are specifically targeted to this domain. To further our understanding of this process, we dissected the ciliary targeting signal (CTS) of Fibrocystin, the product of the gene defective in human autosomal recessive polycystic kidney disease. We show that the CTS is an 18 amino acid motif localized in the cytoplasmic tail. This 18 residue motif is sufficient to target GFP to cilia of ciliated cells. Recent evidence shows the small G protein Arf4 directly binds a sequence in Rhodopsin required for ciliary targeting (Deretic et. al. Proc Natl Acad Sci U S A. 2005). To explore the role of Arf4 in the ciliary

trafficking of Fibrocystin co-immunoprecipiations were carried out with Fibrocystin and Arf4. The CTS of fibrocystin interacts specifically with Arf4 but not other members of the Arf family. Mutation analysis within the CTS of Fibrocystin show the CTS/Arf4 interaction is required for ciliary targeting. To further understand the role of Arf4 mouse ES cells from the Sanger Institute were used to create an Arf4 knockout mouse. Arf4 null mice die embryonically at mid gestation with defects in left-right determination. Current research is focused on determining the role of Arf4 in mouse development and ciliary assembly.

2552/B261

Role of C. Elegans Meckel Syndrome-Related Proteins in Cilia Function.

N. Bialas, P. Inglis, C. Li, M. Leroux; Mol. Biol. and Biochem., Simon Fraser University, Burnaby, BC, Canada

We use *C. elegans* to understand the sensory and signaling functions of cilia, which are complex microtubule-based organelles responsible for sensory and signaling processes and implicated in various human genetic diseases, including Bardet-Biedl syndrome and Meckel syndrome. These and other ciliopathies are collectively characterised by obesity, polycystic kidney disease, retinal degeneration and other ailments. Our functional genomics studies identified numerous novel putative ciliary genes, and we have shown ciliary roles for many of these. Recently, we became interested in 3 evolutionarily-conserved proteins that contain a so-called B9 domain, one of which is associated with Meckel syndrome (MKS1). We uncovered functional interactions between these three proteins in *C. elegans*. All three proteins localise at the base of cilia (basal body), where they are not necessary for the formation of cilia, but instead, are needed for proper insulin signaling. Specifically, disruption of *C. elegans* MKS-1 or MKS-1-related proteins 1 and 2 (MKSR-1 and MKSR-2) leads to an increase in the lifespan of animals that we attribute to impaired insulin signaling. The B9 proteins appear to function upstream of the DAF 2 insulin receptor, and DAF 16 FOXO transcription factor, which upregulates genes required for specifying lifespan. These findings are important, as they suggest that all B9 proteins may be linked to Meckel syndrome or other ciliopathies, and provide new insights into the role of cilia in the insulin signaling pathway. Interestingly, homology searches for B9 domains only reveal matches to the B9 domains found in a group of three orthologues widely distributed in ciliated organisms. However, structure predictions suggest that the B9 domain is related to the C2 domain, which is known to bind calcium and phospholipids, and has been implicated in vesicular trafficking and fusion of vesicles at membranes. Altogether, these data suggest that B9 proteins may regulate the delivery/trafficking of important ciliary cargo to basal bodies, may help the docking of vesicles near basal bodies, or, may assist with the fusion of cargo-bearing vesicles near the ciliary membrane for subsequent incorporation into the organelle.

2553/B262

The Primary Cilium Coordinates Directional Cell Migration.

L. Schneider[1], M. Cammer[2], J. Lehman[3], S. Nielsen[1], I. Veland[1], C. Stock[5], B. K. Yoder[3], A. Schwab[5], P. Satir[4], S. T. Christensen[1]; [1]Department of Biology, University of Copenhagen, Copenhagen, Denmark, [2]Analytical Imaging Facility and Gruss-Lipper Biophotonics Center, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, [3]Department of Cell Biology, University of Alabama at Birmingham, Birmingham, AL, [4]Department of Anatomy and Structural Biology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, [5]Institute of Physiology II, Münster University, Münster, Germany

Objective: Characterization of the primary cilium as a cellular GPS that coordinates directional cell migration and PDGFRα-mediated chemotaxis in fibroblasts. Methods: Cell cultures of NIH3T3 cells, wt and IFT88Tg737Rpw mouse embryonic fibroblasts (MEFs); Immuno Fluorescence Microscopy; Localization of GFP-tagged fusion proteins; SDS PAGE, Immunoprecipitation and western Blotting Analysis; in vitro scratch assay for migration studies; Micropiette assay for chemotaxis analysis; in vivo wound healing of wt and ORPK (IFT88Tg737Rpw) mutant mice. Cells were serum-starved to induce growth arrest and formation of the primary cilium, which emanates from the mother centriole in the centrosome at the cell surface Results: 1) Primary cilia locate predominantly in front of the nucleus and orient in the direction of the leading lamellae of migrating fibroblasts with PDGFRα localizing uniquely to the cilium. 2) Fibroblasts from wt mice with normal primary cilia, acquire chemotactic behaviour when exposed to a PDGF-AA gradient, followed by an increase in migration speed and directional cell movement. In contrast, IFT88Tg737Rpw MEFs that form no or very short cilia, do not show chemotaxis, and do not respond to PDGF-AA. 3) PDGF-AA stimulation leads to activation of PDGFRα in the primary cilium and activation of Akt/PKB at the mother centriole in wt MEFs. These activations are inhibited in IFT88Tg737Rpw MEFs. 4) In the absence of stimuli IFT88Tg737Rpw MEFs have a higher migration speed and translocation rate than wt MEFs in scratch assays. 5) In vivo studies showed that ORPK mutant mice that lack the primary cilium have a significantly reduced rate of wound repair and defects in wound closure compared to wt mice. Conclusions: Our data suggest that PDGFRα signalling in the fibroblast primary cilium regulates centrosomal molecules to monitor cytoskeletal reorganization affecting directional movement during wound healing and developmental processes. In the absence of chemosensory stimuli, signals from the primary cilium may restrain excessive cell migration to prevent uncontrolled and incorrect displacement of cells as seen in invasive cancers and fibrosis.

2554/B263

Mutation in Bardet-Biedl Syndrome Genes Impair Phototaxis in Chlamydomonas.

K. Lechtreck[1], E. C. Johnson[1], T. Sakai[2], M. J. Sanderson[2], M. Ikebe[2], G. B. Witman[1]; [1]Department of Cell Biology, University of Massachusetts Medical School, Worcester, MA, [2]Department of Physiology, University of Massachusetts Medical School, Worcester, MA

Bardet-Biedl Syndrome (BBS) is a cilia-related disorder characterized predominantly by obesity and retinopathy. In humans, 12 genes causing BBS have been identified; 8 of these are conserved in Chlamydomonas. Because of its advantages for both genetic and biochemical studies, Chlamydomonas has great potential to elucidate the function of the BBS proteins in the cilium. Using PCR, we identified insertional mutants defective in the Chlamydomonas BBS1, BBS4, and BBS7 genes. The mutants assemble motile, full-length flagella but lack the ability to orient themselves in a light gradient. The nonphototactic phenotype of the bbs4 mutant was rescued using either the wild-type BBS4 gene or cDNA-based constructs encoding HA- or GFP-tagged BBS4, confirming that the phenotype is caused by the lack of BBS4. Western blotting using anti-BBS4 and anti-HA antibodies showed that BBS4 or BBS4-HA was present in the detergent-soluble fraction of isolated flagella of wild-type and the mutant rescued with the HA-tagged protein, respectively; immunofluorescence microscopy showed that

BBS4 or BBS4- HA co-localized with a subset of intraflagellar transport (IFT) particles in the flagella. TIRF microscopy revealed that BBS4-GFP undergoes IFT at standard IFT velocity but reduced frequency (0.6 vs. 1.3 particles/sec for IFT20-GFP). Compared to the IFT proteins IFT20 and KAP, epitope-tagged BBS4 was present in sub-stoichiometric amounts on western blots of isolated flagella. IFT appeared to be unaffected in the bbs4 mutant. Affinity purification of BBS4-HA from flagellar extracts and sucrose density gradients showed that BBS4-HA is present in a ~12 S complex containing at least four other BBS proteins (BBS1, BBS5, BBS7, and BBS8). Western blots revealed that BBS4 is absent from the flagella of the bbs1 and bbs7 mutants. These data indicate that BBS4 is transported and functions together with other BBS proteins in the flagellum of Chlamydomonas, and is dependent on BBS1 and BBS7 for this transport. 1D- and 2D-electrophoretic analyses revealed several differences between wild-type and bbs4 flagella; current efforts are aimed at determining how the altered protein composition of the bbs4 flagellum results in the phototaxis defect.

2555/B264

Traf3ip Mutant Mice Display Phenotypes Associated with Cilia Dysfunction.

N. Berbari, M. Croyle, B. K. Yoder; Cell Biology, University of Alabama at Birmingham, Birmingham, AL

Primary cilia are solitary appendages found on nearly all mammalian cells, where they serve important sensory and signaling roles. Defects in cilia structure or function are associated with an emerging list of genetic disorders such as, Bardet-Biedl Syndrome, Joubert Syndrome, Meckel Syndrome, Alstrom Syndrome and Polycystic Kidney disease. Cilia disorders, collectively termed ciliopathies, present with a wide range of clinical features including anosmia, retinopathy, mental retardation, infertility, obesity, cystic kidney disease, situs inversus, polydactyly, and heart defects. The broad clinical features observed in ciliopathies are thought to be due to the ubiquitous nature of the primary cilium and the unrecognized roles of cilia in most cell types and tissues. Tumor necrosis factor alpha receptor 3 associated protein 1 (Traf3ip), also known as MIP-T3, has been shown to serve a role in the cilium in various model systems, such as C. elegans and zebrafish. To analyze function of Traf3ip in mammals, we utilized an embryonic stem cell line containing a gene trap insertion into the Traf3ip gene to generate a mouse model. Interestingly, the Traf3ip mutants are not viable and display numerous phenotypes associated with cilia dysfunction, such as polydactyly and heart defects. We hypothesize that these features are due to defects in cilia signaling indicating that traf3ip function may be evolutionarily conserved. Current studies are aimed at elucidating the role of Traf3ip in the mammalian cilium.

2556/B265

Biliary Exosomes Interact with Cholangiocyte Primary Cilia Influencing Intracellular Signaling and Cell Proliferation.

A. Masyuk, C. Ward, S. Gradilone, B. Huang, J. Banales, T. Masyuk, N. LaRusso; Internal Medicine, Mayo Clinic College of Medicine, Rochester, MN

Objective: Exosomes are small, extracellular vesicles present in many biological fluids. They derived from intracellular multivesicular bodies (MVB) and released by a variety of cells. The functional roles of exosomes remain obscure. In some cases, for example in the developing embryo, exosomes interact with primary cilia acting as paracrine messengers. In this study, we tested the hypothesis that exosomes are present in bile where they function as signaling entities affecting cholangiocytes via ciliary-associated mechanisms. Methods: Exosomes were isolated from rat bile by ultracentrifugation and characterized by Western blotting, scanning and transmission electron microscopy. Binding of biliary exosomes to cholangiocyte cilia and the effects of such binding on [Ca2+]i and Erk1/2 signaling and cholangiocyte proliferation were tested on ciliated normal rat cholangiocytes in culture. Results: Biliary exosomes ranging in diameter from 40-100 nm were isolated and fractionated into six major subpopulations. Exosomes were identified by their characteristic “deflated football” shape, the specific MVB markers, CD63 and CD133, and by the presence of the cholangiocyte proteins such as polycystin-1 (PC1), polycystin-2 (PC2) and fibrocystin (FC). Electron microscopy of intact normal rat and mouse livers demonstrated that PC1- and FC-positive exosome-like vesicles surround cholangiocyte primary cilia. Importantly, the number of vesicles surrounding cilia in dilated bile ducts of PCK rats and Pkhd1del2/del2 mice (animal models of Autosomal Recessive Polycystic Kidney Disease) was increased 20-fold. In vitro, isolated biliary exosomes bound to cilia of rat cholangiocytes in a rapid and specific manner. This interaction induced a 2-fold increase in [Ca2+]i and a 65% inhibition of Erk1/2 followed by inhibition of cholangiocyte proliferation in culture by 19%. All effects of biliary exosomes were abolished by pharmacological removal of primary cilia. Conclusions: Bile contains exosomes that influence cholangiocyte intracellular signaling and proliferation by binding to primary cilia. Biliary exosomes and their interaction with primary cilia likely represent a novel extracellular paracrine signaling pathway in liver.

2557/B266

Characterization of Primary Cilia and Their Sensory Function in Human Embryonic Stem Cells.

P. L. Jensen[1,2], A. Awan[1,2], C. Y. Andersen[2], S. T. Christensen[1]; [1]Department of Biolology, University of Copenhagen, Copenhagen Ø, Denmark, [2]Laboratory of Reproductive Biology, University Hospital of Copenhagen, Rigshospitalet, Copenhagen, Denmark

Human embryonic stem cells (hESCs) are potential therapeutic tools and models of early human development. Previously we showed that hESC form primary cilia, which contain critical components of the Hedgehog (Hh) pathway involved in differentiation, including smoothened (Smo) and patched (Ptc)(Kiprilov, Awan et al. 2008. JCB). Stimulation of the Hh pathway resulted in the concerted movement of Ptc out of, and Smo into, the primary cilium, indicating a functional role of the hESC primary cilium in Hh signaling. Here we investigated the localization and activation of signal transduction pathways in primary cilia of a new hESC cell line LRB010 derived from the inner cell mass of a blastocyst in our laboratories. Initially, we show that LRB010 cells grown under feeder-free conditions are indeed undifferentiated human embryonic stem cells, and that they form primary cilia as judged by RT-PCR, western blot and immunofluorescence (IF) microscopy analysis on transcription factors, OCT4, SOX2, and NANOG and with acetylated α-tubulin staining to mark primary cilia. The transcription factors are markers whose interplay is thought to be responsible for the maintenance of the undifferentiated state (Loh et al. 2008. Cell Cycle). Then by IF analysis we demonstrate localization of Wnt signalling components (phospho-β-catenin and Dvl-1) as well as PDGFRα but not PDGFRβ, Tie-2 and polycystin-2 in the primary cilium/centrosome axis of hESC. We also unexpectedly observed OCT4, SOX2, and NANOG along the length of the cilium in a subpopulation of the stem cells. Stimulation of Hh signaling and translocation of Smo to the primary cilium was associated with increased levels of Gli2 activator forms at the tip of the primary cilium. Since Wnt signaling maintains

the self-renewal of hESC (Dravid et al. 2005. Stem Cells) and PDGFR signaling helps maintaining hESC undifferentiated (Pébay et al. 2005. Stem Cells), our results support the idea that the primary cilium coordinates cellular signaling in maintenance of stem cell pluripotentiality, self renewal and in regulation of early differentiation of hESC.

2558/B267

Ankyrin-G Is a Molecular Partner for the Cyclic Nucleotide Gated Channel in Rod Photoreceptor Outer Segments.

K. Kizhatil[1], S. Baker[2], V. Arshavsky[3], V. Bennett[1]; [1]Cell Biology, Duke University Medical Center, Durham, NC, [2]Ophthalmology, Duke University Medical Center, Durham, NC, [3]Ophthalmology and Parmacology, Duke University Medical Center, Durham, NC

The exquisite sensitivity of rod photoreceptors to light depends on the precise localization of the cyclic nucleotide gated channel (CNG) ion channel to the plasma membrane of outer segments, one of the largest known sensory cilia. Here, we report that localization of the CNG channel to the outer segment of rod photoreceptors requires an interaction with ankyrin-G and that the loss of such an interaction leads to retinitis pigmentosa. We first discovered that ankyrin-G and beta-4-spectrin localize to outer segments strongly suggesting the existence of a membrane skeleton. We then found that ankyrin-G binds directly to the rod CNG channel beta subunit (CNG-β1) which is critical for proper channel assembly in the outer segment. Next, we discovered that depleting ankyrin-G in mouse photoreceptors by electroporating plasmid expressing small hairpin RNA drastically reduced levels of the CNG channel but not rhodopsin in outer segments (that were 70 per cent shorter in length than controls). We identified a human mutation of CNG-β1 causing retinitis pigmentosa that abolishes ankyrin-G-binding. This mutation deletes twenty eight amino acids at the carboxyl terminal of the CNG-β1 polypeptide. Using transgenic expression in Xenopus tadpoles, we found that the ankyrin-G binding defective CNG-β1 human mutant protein localized to photoreceptor cell bodies in marked contrast to wild type beta subunits which localized to the rod outer segment plasma membrane. We then discovered that ankyrin-G binding was abolished by mutating just two of the twenty eight amino acids at positions I1237 and L1238 of the CNG-β1 peptide to alanine (IL/AA). Experiments to determine the localization of the more subtle ankyrin-G binding defective IL/AA CNG-β1 mutation in photoreceptors of transgenic Xenopus tadpoles is underway. Together, these results demonstrate that an ankyrin-G-CNG channel interaction is required for maintenance and possibly targeting of the channel in photoreceptor outer segments. This study in addition suggests a role for ankyrin-G in the localizing membrane proteins in primary cilia of other metazoan cells.

2559/B268

Identification of Novel Ciliary G Protein-coupled Receptors and Regulation of Ciliary Localization.

K. Mykytyn, J. Domire; the Ohio State University, Columbus, OH

We recently reported the identification of a ciliary localization consensus sequence in the third intracellular loop of G protein-coupled receptors (GPCRs) and the utilization of this sequence to identify a novel ciliary GPCR. We also reported that the proteins mutated in the human ciliary disorder, Bardet-Biedl syndrome (BBS), are required for the localization of GPCRs to cilia on neurons throughout the brain. Here, we report the identification of additional novel ciliary GPCRs, including dopamine receptor 1 (D1). Interestingly, ciliary localization of D1 is more prominent in BBS neurons, suggesting the BBS proteins are also involved in the regulation of GPCR trafficking out of cilia. Furthermore, in wildtype neurons D1 ciliary localization increases in response to treatment with D1 agonists. Our results suggest a novel role for the BBS proteins in the regulation of GPCR trafficking from the cilium and indicate that GPCR ciliary localization is dynamic in response to receptor activation.

2560/B269

Role of the Ciliary Motor Protein KIF3A in Epithelial Cell Polarity.

C. Boehlke[1], B. Buchholz[2], M. Herbst[1], S. Braeg[1], H. Voelker[1], K. Eckardt[2], G. Walz[1], W. Kuehn[1]; [1]Nephrology, University Clinical Center, Freiburg, Germany, [2]Nephrology, University Medical Center, Erlangen, Germany

Dysfunction of primary cilia, hair like structures projecting from the surface of most cells, have lately been involved in a broad spectrum of diseases such as retinal degeneration, polycystic kidney disease and obesity. An evolutionary conserved intraflagellar transport system helps sustain ciliary strucure and function. Anterograde ciliary transport is maintained by kinesin II and mutation of the kinesin II subunit KIF3A in mice leads to the loss of tubular geometry and cyst formation. In rat hippocampal neurons KIF3A interacts with Par-3 to mediate the outgrowth of axons, suggesting that KIF3A might have extra-ciliary functions in epithelial polarity. Abrogating ciliogenesis by inducible knock-down of KIF3A in MDCK cells resulted in normal localization of apical-basal polarity markers such as zo-1, E-cadherin and beta-catenin. Calcium-switch experiments however revealed delayed tight junction assembly after depletion of KIF3A. When challenged to grow in a collagen I matrix, KIF3A depleted cells lost the ability to polarize into three-dimensional structures and to form extensions after HGF treatment. Interestingly, cells with PKD2 depletion after inducible knock-down formed normal spherical strucutes in collagen, suggesting that the role KIF3A on three-dimesional polarization is independent of the cilium. Scratch induced wound healing of unciliated cells in 2D culture resulted in gross impairment of migration speed after KIF3A depletion, but normal scratch- and growth factor-induced activation of ERK1/2 and Rac1. Immunofluorescence revealed mislocalization of the golgi apparatus and Par-3 in migrating cells. Our findings uncover a novel role of KIF3A in the polarization of epithelial cells. They imply the necessity of kinesin II for the formation of three dimensional epithelial structures such as tubules, independently of cilia.

2561/B270

Polarity and Migration of Wound Repair Cells of the Inner Lobe Surface of the Ctenophore Mnemiopsis Leidyi.

M. B. Dodson, A. Moss; Auburn University, Auburn, AL

Our lab is currently developing the ctenophore, Mnemiopsis leidyi, as a model system to study the cellular and molecular bases of rapid, scar-free wound healing. Wounds of up to 3 mm long were generated with an iridectomy knife in the ciliated lobular epithelium. We examined epithelial and mesogleal wound repair cells of the lobe inner surface. Within a few minutes of wounding, many thousands of 10-15 µm diameter, multipolar, bipolar and tripolar mesogleal cells migrated to the edge of the wound site. As described previously for inter-row wound repair (Dodson/Moss, 2007 ASCB), both bipolar and multipolar cells were observed to crawl on the under surface of the epithelium to

the cut edge, where they formed masses which accumulated for several minutes before sending forth processes to span the wound gap. In addition, pre-existing epithelial cells elongated and moved in a directed manner toward the wound site. Epithelial cells exhibited distinct polarity, with a flattened, elongated appearance and, trailing nucleus. Such cells displayed a flattened slightly spread leading edge pointed in the direction of the wound. Although all of the cells of the cut edge were observed to actively migrate, cells were observed to differentially crawl past each other. Examination of the cavity formed in the mesoglea by the wound revealed that individual compound cilia were aligned with their distal tips pointing into the wound. Cirri of migrating cells were non-motile and often oriented at 180 degrees to their normal orientation (resting cilia of the inner lobe surface normally come to rest with the end of the power stroke directed toward the mouth - in wounded epithelia they unerringly pointed toward the wound cavity). Cirri continued to point into the wound cavity until the edge of the wound was almost entirely closed. Upon closure, the elongated shape of the cells rapidly became more rounded, while the cells under the wound dispersed. Analysis of the expression of rapidly migrating, wound-repair cells has not, to date, revealed significant differential expression of cytoskeletal, matrix or membrane protein genes.

2562/B271

Inversin Is Required for Directional Cell Migration in Fibroblasts.

I. R. Veland[1], L. Eley[3], S. F. Pedersen[1], J. Goodship[3], A. Schwab[2], S. T. Christensen[1]; [1]Institute of Biology, University of Copenhagen, Copenhagen, Denmark, [2]Institute of Physiology II, University of Muenster, Muenster, Germany, [3]Institute of Human Genetics, University of Newcastle, Newcastle upon Tyne, United Kingdom

Cell migration is necessary for many physiological processes including wound healing, immunity, and embryonic development. Recently, we showed that PDGFRαα-mediated chemotaxis is coordinated by the primary cilium in fibroblasts through activation of the sodium-proton exchanger NHE1 at the leading edge (Schneider et al., submitted). *Tg737^{orpk}* MEFs that have short or no primary cilia are unable to respond to PDGF-AA, but display increased and uncontrolled migratory activity. Since Inversin is a ciliary protein (e.g. Morgan et al. 2002 Hum Mol Genet 11:3345) and implicated in non-canonical Wnt signaling (Simons et al. 2005 Nat Genet 37:537) preceding cell migration (reviewed in Christensen et al. 2008 submitted) we used wt and *inv-/-* mouse embryonic fibroblasts (MEFs) to investigate the role of Inversin in cell migration. As opposed to *Tg737^{orpk}* MEFs, *inv-/-* MEFs form primary cilia but are largely immobile. Tracking of cell movements in wound healing assays during a 6h time course showed that migration into the wound was more than 10 times lower in *inv-/-* MEFs as compared to wt MEFs, whereas general displacement was 7 times lower. In wt MEFs the average velocity was 38.51±10.29 µm/sec while only 10.6±2.68 µm/sec in *inv-/-* MEFs. Neither PDGF-AA nor Wnt5a, which are ligands known to stimulate cell migration, could restore the levels of displacement and velocity to that of wt MEFs, indicating that both the PDGFRα and non-canonical Wnt signaling pathways are disrupted in *inv-/-* MEFs. In wt cells primary cilia orient parallel to the migration path and in front of the nucleus and this is associated with formation of F-actin-based protrusive structures, such as lamellipodia at the leading edge. In contrast, cilia are randomly positioned in *inv-/-* MEFs, which maintain an unpolarized actin cytoskeleton with extensive stress fibers and no lamellipodia. Preliminary evidence also shows that *inv-/-* MEFs have altered levels of actin cross linking proteins, such as ezrin. Further, Cofilin in *inv-/-* MEFs may have an increased phosphorylation on its serine in pos. 3, which reduces F-actin polymerization. These data support the hypothesis that Inversin is a central regulator of cell polarity in cell migration.

2563/B272

Human Ciliated Airway Epithelia Are Chemosensory and Contain Taste Receptors That Respond to Bitter Compounds.

A. Shah[1], Y. Ben-Shahar[2], T. O. Moninger[1], P. H. Karp[1], M. J. Welsh[1,3]; [1]Internal Medicine, University of Iowa, Iowa City, IA, [2]Biology, Washington University in St. Louis, St. Louis, MO, [3]Howard Hughes Medical Institute, Iowa City, IA

Defense of the human airway is critical for survival. The multiple defense mechanisms include the detection of noxious agents by pattern recognition molecules such as the toll-like receptors (TLR). On the tongue, bitter taste serves as a defense to avoid the ingestion of harmful substances. We hypothesized that the airways might contain a detection mechanism that also involves taste receptors that were previously thought to be specific for gustation. To test this hypothesis, we used primary cultures of differentiated human airway epithelia. Using microarray analysis, we detected ten taste receptor transcripts, all were bitter receptors, and none were for sweet or umami. Immunofluorescence identified Tas2R, the bitter taste receptor, only in ciliated epithelial cells, and not in goblet or non-ciliated cells. We obtained similar results from fresh tissue samples. Tas2R localized either to the base of motile cilia or to the apical membrane. Furthermore, α-gustducin, the related G-protein, and TrpM5, the taste-transduction channel, localized to the motile cilia. PLC-β2, the downstream taste activator, localized to the apical region of ciliated cells. Denatonium, a bitter compound, induced a dose-dependent transient increase in intracellular calcium. Thujone, another bitter compound, also gave a dose-dependent, but more prolonged calcium response. To test the physiological consequences, we examined the effect of denatonium on cilia and on transepithelial transport. Denatonium increased cilia beat frequency and inhibited transepithelial chloride transport. Our results indicate that ciliated airway epithelial cells contain the machinery to detect bitter compounds and thus, they are chemosensory. These findings suggest an evolutionarily conserved defense mechanism involving bitter taste receptors on both the tongue and the ciliated epithelial cells of the intrapulmonary airways.

2564/B273

Primary Cilia Act as Biosensors to Regulate Production of Cerebrospinal Fluid.

K. Narita, T. Kawate, S. Takeda; Anatomy and Cell Biology, University of Yamanashi, Chuo, Japan

Cilia are projections from the cell surface supported by a microtubular cytoskeleton called the axoneme, and classified into two subgroups, conventional and primary cilia. The primary cilia are principally considered as biosensors for monitoring the extracellular environment. Defects in cilia are often associated with hydrocephalus, for which contributions of conventional cilia expressed in ependymal cells in the flow of cerebrospinal fluid (CSF) have been well described. On the other hand, we speculated that malfunctions of cilia lead to overproduction of CSF by choroid plexus epithelial cells (CPECs) expressing primary cilia. However, the molecular mechanisms of hydrocephalus due to malfunctions of primary cilia have not yet been clarified. In this study, we investigated the function of primary cilia in CPECs to regulate CSF production. CPECs were isolated from porcine brain and cultured to confluent monolayers. These cells expressed

clusters of primary cilia on the apical surface. Deciliation by chloral hydrate increased the intracellular cAMP levels, as demonstrated by a competitive immunoassay. Characterizations of CPEC monolayers prepared on Transwell membranes indicated that deciliated CPECs had higher activities in the basolateral-to-apical fluid transcytosis than untreated control. Furthermore, immunostaining revealed that the primary cilia possessed a homolog of neuropeptide FF (NPFF) receptor 2. Treatment of the cells with NPFF resulted in decreased intracellular cAMP levels and fluid transcytosis, both of which were antagonized by deciliation. These results collectively demonstrated that primary cilia in CPECs regulated intracellular cAMP levels and in turn modulated the basolateral-to-apical fluid transcytosis. All in all, primary cilia may play an important role in regulating the production of CSF via NPFF-mediated intracellular signaling mechanism.

2565/B274

Stable and Prolonged Contacts Form between Cilia of Adjacent Cells.

C. Ott[1], P. Sengupta[1], N. Elia[1], L. Case[2], J. Lippincott-Schwartz[1]; [1]Cell Biology and Metabolism, NICHD, NIH, Bethesda, MD, [2]NIH-UNC Graduate Partnership Program, NIH, Bethesda, MD

Primary cilia have been described as antennae because they often project away from the cell surface and they are able to receive signals (both chemical and mechanical) from the extracellular environment. Imaging this important organelle is essential to developing a better understanding how it functions. By imaging live polarized epithelial cells we found that cilia can make direct contact with cilia of adjacent or nearby cells. Imaging live cells expressing different cilia-localized fluorophores revealed that the bridges are composed of cilia from individual cells: they are not a continuation from one cell to the next. Cilia bridges can be stable over many hours. Trypsin and other protein disrupting treatments have not disrupted the cilia-cilia adhesion. These findings suggest that cilia may do more than just passively sense the environment. They may be able to initiate and mediate cell-cell communication through direct contact.

2566/B275

Articular Chondrocytes Express Connexin 43 Hemichannels and P2 Receptors - a Putative Mechanoreceptor Complex Involving the Primary Cilium?

S. McGlashan[1], M. M. Knight[2], M. Garcia[2], C. Poole[3], C. Jensen[1]; [1]Anatomy with Radiology, University of Auckland, Auckland, New Zealand, [2]Medical Engineering, Queen Mary University of London, London, United Kingdom, [3]Medical and Surgical Sciences, University of Otago, Dunedin, New Zealand

Mechanical loading is essential for the health and homeostasis of articular cartilage. Previous studies have demonstrated that cyclic compression of chondrocytes up-regulates proteoglycan synthesis via an intracellular Ca2+ signalling pathway, mediated by the release of ATP. However, the mechanism(s) of ATP release has not been elucidated. The present study examined the expression of the putative mechanosensitive ATP release channel, connexin 43 and its expression on the chondrocyte primary cilium. In addition we characterised the expression of a range of purine receptors through which ATP may activate downstream signalling events. Bovine articular chondrocytes were isolated by sequential enzyme digestion and seeded in agarose constructs. To verify the presence of functional hemichannels, Lucifer Yellow (LY) uptake was quantified following treatment with a hemichannel agonist (EGTA) and antagonist (flufenamic acid). LY uptake was observed in 45% of chondrocytes, increasing to 83% following EGTA treatment ($p<0.005$). Treatment with flufenamic acid significantly decreased LY uptake to less than 5% with or without EGTA. Using confocal microscopy, we showed that approximately 50% of bovine chondrocyte primary cilia were decorated with connexin 43. Human chondrocytes in situ also expressed connexin 43 hemichannels, as shown by immunolabelling of both the intracellular and extracellular domains. However expression was confined to the 200µm of the tissue closest to the articular surface. P2X4, P2X7, P2X2, P2Y1 and P2Y2 purine receptor subtypes were also detected in human articular cartilage. In conclusion, functional hemichannel expression by articular chondrocytes may represent a mechanism through which mechanical loading activates ATP release as part of a purinergic mechanotransduction pathway. Human articular chondrocytes express a range of P2X and P2Y receptors allowing them to respond to extracellular ATP. Connexin 43 expression on the chondrocyte primary cilium suggests the possible involvement of the cilium in this mechanotransduction pathway.

2567/B276

The Cause and Effects of Synchronisation between Bull Sperm Flagella.

D. M. Woolley[1], R. F. Crockett[1], W. D. Groom[1], S. G. Revell[2]; [1]Physiology and Pharmacology, University of Bristol, Bristol, United Kingdom, [2]Genus Breeding Ltd, Ruthin, United Kingdom

Flagellar synchronisation can be observed between bull spermatozoa when they swim in a viscous medium, confined to a glass surface. The process is of interest in understanding the regulation of beating frequency. The simple method of dark-field video-microscopy has shown that exact and persisting synchrony occurs only when the sperm heads lie in superposition and are tightly coupled, mechanically. Viscous coupling between the flagella is not by itself sufficient to establish synchronisation. Immediately following synchronisation, the two conjoined spermatozoa show rises in beat frequency, wave velocity and swimming speed: that is, in nearly all cases, the new values are greater than those shown by either of the two singleton spermatozoa. This result, particularly the rise in beat frequency, was unexpected. There are reasons for seeing the primary change as a rise in wave velocity, via a decreased viscous resistance to bend propagation. The rise in beat frequency is seen as consequential to the rise in wave velocity, mediated by a feedback process. The rise in swimming velocity implies that the increased propulsive thrust is not balanced by a proportionate increase in the drag experienced by the sperm heads. A major contributing factor here is that the conjoint sperm heads become subtly re-orientated, such that there is a smaller area of contact with the cover slip; this alternative, stable orientation is possible because the plane of beating normally diverges slightly from the plane in which the sperm head is flattened. Some general questions are raised. One is whether mechanical coupling between the basal bodies of cilia might similarly be necessary for establishing their coordination. Another is whether a minority of bull spermatozoa, that become attached together and synchronised in the confines of the epididymis, might have an advantage in penetrating the mucus of the female tract.

2568/B277

Force Generation and Dynamics of Individual Cilia under External Loading.

V. Swaminathan[1,4], D. Hill[2,4], E. O'Brien[3,4], R. Superfine[3,4]; [1]Curriculum in Applied Sciences and Engineering, UNC- Chapel Hill, Chapel Hill, NC, [2]Cystic Fibrosis/Pulmonary Research and Treatment Center, UNC- Chapel Hill, Chapel Hill, NC, [3]Department of Physics and Astronomy, UNC- Chapel Hill, Chapel Hill, NC, [4]Virtual Lung Project, UNC- Chapel Hill, Chapel Hill, NC

Determining the force generated by individual human lung epithelial cilia is critical for predictive modeling of many physiological functions including the ability of cilia to clear pathogens and particulates via transport of mucus. Such a measurement also allows a reasonable calculation of the force produced by each dynein motor during the active stroke, which is of interest to understanding motor function within all cellular systems. We covered 2.8 um magnetic beads with gold, except for a small area of streptavidin on each bead, and used biotinylated wheatgerm agglutinin to bind the beads to cilia. We recorded bead motion while we applied forces on the bead via magnetic fields generated by a 3-Dimensional Force Microscope. We found that forces up to those sufficient to reduce beat amplitude by 85% did not alter the cilia beat frequency, and thus the axoneme behaved as an internally driven system throughout its beat cycle. Forces in excess of 160 pN were required to reduce the beat amplitude below 15%. We cast these results into a simple one dimensional model of axoneme dynamics, which yields an effective internal motor force of 62pN ± 14pN, or ~ 6pN per active dynein.

Nerve Cell Cytoskeleton (2569 – 2590)

2569/B278

Polarized Distribution of Kinesin-5 in Growth Cones Regulates Selective Microtubule Invasion during Turning.

V. Nadar, A. Ketschek, K. Myers, G. Gallo, P. Baas; Department of Neurobiology and Anatomy, Drexel University College of Medicine, Philadelphia, PA

Growth cones typically can be divided into three regions based on cytoskeletal organization: a microtubule (MT)-rich Central (C-) domain, an actin-rich Peripheral (P-) domain and the Transition (T-) zone between these two domains. Selective invasion of MTs on one side of the growth cone into the P-domain from the C-domain is critical for the growth cone to turn in that direction. Previous studies mainly focused on the role of MT dynamics in regulating the polarization of the MT array during growth cone turning. Recent studies from our laboratory suggest that forces generated by molecular motor proteins are also critical determinants of the distribution of MTs in growth cones. Here we report a novel role in growth cone turning for kinesin-5, a homotetrameric motor protein best known for its role in mitosis. In growth cone turning assays, inhibition of kinesin-5 in cultured neurons caused MT distribution to become less polarized in growth cones, with MTs invading all regions of the P-domain. As a result, the growth cones grew past cues that would normally have caused them to turn. Immunostaining for kinesin-5 revealed an enrichment of kinesin-5 specifically in the T-zone. Immunostaining with a phospho-specific antibody revealed an enrichment of phosphorylated kinesin-5 on the side of the growth cone showing the least MT invasion into the P-domain. This is notable because binding of kinesin-5 to MTs is regulated by phosphorylation at the site recognized by this antibody. Collectively, these results are consistent with a scenario by which kinesin-5, when bound to MTs, acts as a gatekeeper that restricts the entry of MTs into the P-domain. We speculate that signals relevant to growth cone turning can influence the polarization of the MT array by causing more kinesin-5 to be phosphorylated on the side of the growth cone opposite to the direction of turning.

2570/B279

Myosin II Activity Is Necessary for Growth Cone Turning at the Chondroitin Sulfate Proteoglycan Boundary.

L. Santiago, Y. Katagiri, H. Geller; National Institutes of Health, Bethesda, MD

Chondroitin sulfate proteoglycans (CSPGs) are major inhibitors of axonal growth in the injured spinal cord. Our overall goal is to understand the role of actin, microtubules, and biomolecular motors in the behavior of the growth cone at a boundary containing CSPGs. When neuronal growth cones encounter a boundary containing CSPGs, they either stop at the boundary or turn away from it. Although the intracellular mechanisms involved in growth cone turning remains unknown, the molecular motor myosin II has been implicated in the growth cone turning behavior at extracellular matrix boundaries, as observed when axons growing in laminin encounter a boundary containing polyornithine. We therefore investigated whether myosin II was involved in growth cone turning at CSPG boundaries. Postnatal mouse cerebellar granule neurons (CGNs) were seeded onto poly-lysine (PLL)-covered coverslips containing spots with immobilized chicken CSPGs mixed with Texas red (CSPG concentration ranged of 0-5 micrograms/ml). Myosin II activity was inhibited by adding the pharmacological agent blebbistatin at concentrations ranging from 0-50 μM. 24 hours post- treatment the cells were fixed with paraformaldehyde, stained with antibodies against βIII-tubulin, and the number of axons crossing the boundary was determined. Inhibition of myosin II activity resulted in a significant increase in the percentage of axons crossing from the PLL region into the CSPG spots as compared to controls containing dimethyl sulfoxide (DMSO). The response of CGNs at the boundary with CSPGs was dependent on the concentration of blebbistatin utilized in the culture media, with the maximum crossing observed at 50 μM. Moreover, while the growth cone turning response was significantly reduced by blebbistatin at the lowest concentrations of CSPGs tested, inhibition of myosin II activity was not sufficient to eliminate turning at the highest concentration of CSPGs tested. Our results suggest that the activity of myosin II is essential for growth cone navigation at boundaries containing inhibitory substrates.

2571/B280

Laminin and Serotonin Cooperate to Promote Neuronal Growth Cone Advance.

X. Zhang, D. Van Goor, P. Forscher; MCDB, Yale University, New Haven, CT

Laminin stimulated neurite outgrowth is dependent upon integrin function and Rac1 activation. Serotonin has been described as both a neurotransmitter involved in learning and memory, as well as a neuromodulator with effects on neurite outgrowth. We have previously shown that in a constitutively-active Rac background, serotonin treatment results in both calcium release and growth cone spreading on poly-L-lysine substrates. Here we show that Aplysia bag cell neurons cultured on a laminin substrate display increased rates of neurite outgrowth in an integrin and Rac dependent manner. Interestingly, bath treatment with serotonin further increased neurite outgrowth rates, an effect that is coincident with calcium mobilization throughout the growth cone. Inhibitors of phospholipase C or the IP3 receptor block these responses

without affecting either baseline calcium levels or basal neurite outgrowth rates. In contrast, inhibitors of calcineurin, a key member of calcium signaling pathways, blocked only the increased rates of neurite outgrowth and not the serotonin evoked calcium release. Taken together, these results suggest that the extracellular matrix (i.e. growth/culture substrate) and soluble cues (i.e. serotonin) combine to determine growth cone behaviors. Cytoskeletal dynamics underlying these effects are currently under investigation.

2572/B281

Rac Mediates apCAM-Induced Adhesion Formation Early in Targeted Growth Responses of Aplysia Growth Cones.

K. Buck, V. Schoonderwoert, X. Zhang, P. Forscher; Molecular, Cell and Developmental Biology, Yale University, New Haven, CT

Migrating growth cones create adhesions in order to generate traction forces during neurite guidance and outgrowth. In Aplysia neurons, experimentally restrained beads coated with the cell adhesion molecule apCAM induce the formation of adhesion sites in a spatially restricted manner. Here we present evidence that Rac is a critical early regulator of adhesion formation. Substrate evoked growth proceeds through a defined sequence of spatially coordinated events: apCAM crosslinking and actin polymerization occur at the bead target during the latency phase, followed by force generation and central domain advance towards the target during the growth phase. Since Rho and Rac can exhibit dynamic antagonism, and our previous findings implicate Rho signaling in contractility at the back of the growth cone, we examined a role for Rac signaling at the front of the growth cone during targeted growth responses. We report that Rac protein, but not Rho, accumulates in a force-dependent manner at target sites during the latency phase. Pharmacological inhibition of Rac activity did not drastically affect growth cone morphology, but did block apCAM bead-induced actin assembly and Rac protein accumulation at the target site during the latency phase. Importantly, loss of target adhesion by Rac inhibition also precludes central domain advance, attenuating the ability of growth cones to mount completed targeting responses. Rac is a known component of the NADPH oxidase (NOX) complex, which generates reactive oxygen species (ROS) for signaling. We observed accumulation of NOX components gp91 and p22phox at bead target sites in a Rac- and force-dependent manner. Interestingly, the efficiency of growth responses, but not adhesion assembly, is reduced by experimental dampening of ROS signaling. Our results suggest that Rac plays a pivotal role in establishing a localized actin adhesion site in the periphery during the latency phase, and that downstream ROS signaling may modulate timing or efficiency of the growth phase.

2573/B282

JNK1 Regulates the Rate of Neuronal Migration via SCG10.

N. Westerlund[1], B. Björkblom[1], T. Tararuk[1], T. Kallunki[2], M. Courtney[3], E. Coffey[1]; [1]Turku Centre for Biotechnology, Åbo Akademi and Turku University, Turku, Finland, [2]Danish Cancer Society, Copenhagen, Denmark, [3]A.I. Virtanen Institute, Kuopio, Finland

Appropriate migration of neurons is an important feature of brain development, yet negative regulatory mechanisms have remained obscure. Here we show that JNK1 is highly active in developing forebrain. Inhibiting JNK activity using pharmacological and compartment-targeted, dominant-negative inhibitors induces a striking increase in neuronal migration rate both in culture and in vivo. Moreover, radial migration is accelerated in jnk1-/- mice. The JNK1 substrate SCG10 is a microtubule regulatory protein that is central to this mechanism. Expression of a JNK1-site phosphomimetic mutant of SCG10 restores normal migration in JNK1-/- brains. We find that tubulin levels are rate-limiting for neuronal migration and that the JNK1 phosphorylation sites of SCG10 regulate both tubulin solubility and neuronal migration. These data indicate that phosphorylation of SCG10 by JNK1 provides a mechanism to negatively regulate migration during brain development.

2574/B283

Myosin II Is Required for Sensory Axon Extension on Laminin in the Absence of Nerve Growth Factor.

A. Ketschek, G. Gallo; Neurobiology & Anatomy, Drexel University College of Medicine, Philadelphia, PA

External cues from the surrounding environment are responsible for guiding the growth of axons during development and regeneration. Axon extension occurs through a three-step process sequentially involving protrusion and substratum-attachment of filopodia and lamellipodia, followed by engorgement of the growth cone by microtubules and organelles, and finalized by the consolidation of a new stretch of axon shaft behind the advancing growth cone. Myosin II is a mechanoenzyme that generates contractile forces on actin filaments and is thought to regulate multiple aspects of the process of axon extension. Using Embryonic Day 7 chicken DRG neurons, which on Laminin (LN) can extend axons independent of Nerve Growth Factor (NGF), we report that in the presence of NGF and LN, inhibition of myosin II results in a decrease in the rate of axon extension and an increase in axonal protrusive activity. In the absence of NGF and the presence of LN, inhibition of myosin II blocks axon extension and protrusive activity in sensory neurons. Addition of NGF restored axon extension and protrusive activity. In the presence of NGF and LN, inhibition of myosin II does not affect microtubule content. However, in the absence of NGF and the presence of LN, inhibition of myosin II results in a loss of microtubule content in the growth cone and axon shaft. Addition of NGF partially recovers microtubule content after inhibition of myosin II. Addition of NGF to NGF-naïve neurons increased microtubule content in the growth cone and axon shaft. These data demonstrate that in the absence of NGF signaling sensory axon extension on LN is strictly dependent on myosin II activity and that NGF signaling partially relieves this strict dependence.

2575/B284

Overexpression of Neuronal Intermediate Filament Peripherin Induced Neuronal Death in PC12 Cells.

W. Lee, Y. Chen, C. Chien; Anatomy and Cell Biology, College of Medicine, National Taiwan University, Taipei, Taiwan

Peripherin, a ~57kD neuronal intermediate filament protein, plays a crucial role in pathogenesis of ALS. However, the exact degenerative pathway of cell death involved of peripherin in ALS pathogenesis is unclear. To determine the mechanism of neurodegeneration caused by peripherin, the cDNA of rat peripherin tagged with EGFP was transfected into a rat adrenal pheochromocytoma cell line PC12 that responds to nerve growth factor (NGF) by induction of the neuronal phenotype. After G418 selection, 2 stable cell lines were established. The stable cell line was named pEGFP-Peripherin cells. pEGFP-Peripherin cells were induced by NGF and examined for neurodegeneration by cell survival rate assessment, western blot, immunocytochemistry and conventional electronic microscopy. Peripherin overexpression led to the formation of cytoplasmic aggregation and then causes cell death after NGF induction. Western blot analysis showed the caspase-3, caspase-9 and caspase-12 activation in pEGFP-Peripherin cells. Furthermore, TUNEL positive reaction could be detected in pEGFP-Peripherin cells at

8 day of NGF induction. Abnormal aggregations of neuronal intermediate filaments were found in the cytoplasm ultrastructurally. Besides, abnormal mitochondria, endoplasmic reticulum with swelling pattern and multi-layer autophagosomes could be found in pEGFP-Peripherin cells at 8 day of NGF induction. To sum up, these results suggested that the cytoplasmic aggregation caused by peripherin overexpression may impair mitochondria and endoplasmic reticulum subsequently induced neuronal death.

2576/B285

Probing Aplysia Growth Cone Mechanotransduction Using Holographic Optical Tweezers.

C. O. Mejean[2], A. W. Schaefer[1], P. Forscher[1], E. R. Dufresne[2]; [1]MCDB, Yale University, New Haven, CT, [2]Mechanical Engineering, Yale University, New Haven, CT

Growth cones generate traction forces during neurite outgrowth, however, how force magnitude is adjusted to match compliance of the local environment is not well understood. Indeed, in Aplysia neurons, acute periods of growth cone advance can be triggered by microspheres coated with the cell adhesion molecule (apCAM), if the beads are physically restrained on the cell surface to permit development of traction force. The growth response involves apCAM clustering which induces signaling thereby promoting actin recruitment and new actin assembly at the bead site. Subsequently, linkages are formed between the extracellular environment and myosin dependent filamentous actin flow. Here, we report the design and development of a Holographic Optical Tweezers (HOT)-system designed to mimic physiological substrates using two dimensional arrays of apCAM coated microspheres. We implemented a software based control system with novel feature recognition and hologram calculation algorithms to control the motion and location of numerous microspheres with high spatial and temporal precision. In addition forces can be controlled and measured simultaneously at multiple locations. We use this technology to manipulate apCAM-coated beads to investigate mechanochemical responses of growth cones. When multiple apCAM-coated beads are restrained, transient physical coupling with the underlying moving actin cytoskeleton is observed. Beads placed at different sites on the growth cone using the same restraining force exhibit several apparently uncorrelated responses including: time to flow coupling, motion directionality and force exerted by the growth cone. Currently, we are investigating if there is a correlation between apCAM density, actin recruitment and biophysical characteristics of the response observed.

2577/B286

New Turnover Algorithm Demonstrates Actin Depolymerization Is a Distributed Process in Aplysia Bag Cell Growth Cones.

D. W. Van Goor, P. Forscher; MCDB, Yale University, New Haven, CT

Regulated actin depolymerization is critical to growth cone function. Automated computer analysis has been applied to fluorescent speckle microscopy (FSM) to measure this process in live cells. All turnover algorithms, whether utilizing speckle appearance/disappearance or conservation of mass principles, are ultimately grounded in measuring intensity changes. Here we present evidence that, contrary to currently held assumptions, freely diffusing, labeled monomer makes significant contributions to the intensity of a FSM image. Ratiometric processing enables the removal of this intensity from raw actin images, yielding intensity profiles derived solely from incorporated, labeled monomer. We have incorporated this image processing technique into a new algorithm for measuring the net kinetics of the actin cytoskeleton and applied it to define steady state dynamics in the growth cone. Established speckle tracking algorithms are used to monitor regions of the actin meshwork over time; conservation of mass principles are applied to intensity changes to extract relative turnover rates. Accuracy was verified by direct comparison of predicted actin densities with actual values measured from retrospective immunocytochemistry images. Based upon our results, a picture of the growth cone actin cytoskeleton emerges where polymerization is a localized process that occurs mainly at the leading edge and in intrapodia, also referred to as dorsal ruffles. Depolymerization is a distributed process that occurs throughout the peripheral domain and extends into the central domain. This picture contrasts with current speculation that depolymerization occurs primarily at the transition between the actin dominated periphery and microtubule dense central regions.

2578/B287

The Neural Cell Adhesion Molecule Associates with and Signals through P21-Activated Kinase 1 to Regulate Neuronal Growth Cone Morphology.

S. Li, Y. Chernyshova, I. Leshchyns'ka, V. Sytnyk, M. Schachner; Universitäts-Krankenhaus Eppendorf, Zentrum für Molekulare Neurobiologie, Hamburg, Germany

The neural cell adhesion molecule (NCAM) plays important roles in regulating neuronal morphology, growth and migration. However, the various ways by which NCAM evokes its cellular responses are not completely understood. We identify p21-activated kinase 1 (Pak1) as a new binding partner of the intracellular domain of NCAM. Clustering of NCAM on growth cone membrane induces hyperphosphorylation of Pak1 and its effectors LIM kinase 1 and cofilin. Phosphorylation state, kinase activity and levels of membrane associated Pak1 are abnormal in brains of NCAM-deficient mice. Expression and phosphorylation of LIM kinase 1 and cofilin are also dysregulated in the mutant. NCAM enhances Pak1 activity by promoting formation of Pak1-Cdc42-PIX complexes in lipid rafts. Increased levels of active non-phosphorylated cofilin in NCAM-deficient brains cause excessive actin depolymerization which leads to morphological abnormalities of growth cones in vitro, resulting in enlargement of growth cones, and inhibition of filopodia formation. Our results contribute to an understanding of the molecular mechanisms underlying NCAM-mediated cytoskeletal reorganization and neurite outgrowth.

2579/B288

Semaphorin 3A Induces F-actin Dependent Retrograde Redistribution of Mitochondria in Distal Axons.

N. Kulkarni[1], G. Gallo[2]; [1]Drexel University, Philadelphia, PA, [2]Drexel College of Medicine, Philadelphia, PA

We previously reported that Semaphorin 3A (Sema3A), a repellant guidance cue, generates an intra-axonal system of F-actin bundles that act as the substratum for myosin-II driven axonal retraction. Previous reports identified effects of guidance cues on organelle transport. Thus, apart from the direct action of Sema3A on the cytoskeleton, it is pertinent to understand whether guidance cues modulate the intra-axonal transport of organelles, such as mitochondria. We investigated the mechanisms of mitochondrial redistribution in Sema 3A treated axons. Using embryonic chicken dorsal root ganglion neurons, we found that Sema 3A treatment induced retrograde redistribution of mitochondria

in the distal axon. Experiments blocking Sema3A induced axon retraction, using the myosin II inhibitor blebbistatin, revealed that the mitochondrial redistribution was independent of axon retraction. These results indicate that mitochondrial redistribution occurs independent of bulk cytoplasmic flow and myosin II activity during axonal retraction. We next tested whether Sema3A induced mitochondrial redistribution was dependent on the Sema3A induced axonal F-actin bundles. Cytochalasin D (CD), an inhibitor of actin polymerization, prevented intra-axonal F-actin bundle formation in response to Sema3A treatment. We observed that blocking the formation of Sema3A-induced axonal F-actin bundles blocked the retrograde redistribution of mitochondria normally induced by Sema3A. CD treatment alone did not alter the net distribution of mitochondria in control axons. Collectively, these experiments suggest that the action of Sema3A in redistributing mitochondria is independent of myosin II mediated axon retraction, but is dependent on the formation of axonal F-actin bundles. We propose that in the context of the response to Sema3A, mitochondria may use axonal F-actin bundles as a substratum for transport. Alternatively, the bundles may be required for the orchestration of mitochondrial movements but not serve as a substratum.

2580/B289

PI3K-Akt Pathway Mediates NGF-induced Axonal Filopodia Formation via F-actin Patch Formation.

A. Ketschek, G. Gallo; Neurobiology & Anatomy, Drexel University College of Medicine, Philadelphia, PA

External cues from the surrounding environment are responsible for guiding the growth of axons, as well as establishing neuronal connections via collateral sprouting. Contact with Nerve Growth Factor (NGF)-coated beads induces filopodia formation along the axon shaft of sensory neurons (Gallo and Letourneau 1998). We have previously shown that spontaneously formed F-actin patches are precursors to protrusive activity in the distal axon (Loudon et al 2006). Inhibition of PI3K blocked the formation of F-actin patches and subsequently blocked filopodia formation in axons (Orlova et al 2007). We now report that NGF increases the frequency of F-actin patch formation and subsequently filopodia formation in axons of chicken sensory neurons. Activation of PI3K with a cell permeable peptide increases F-actin patch formation while inhibition of PI3K using LY294002 blocks NGF-induced F-actin patch formation and filopodia formation along the axons. Treatment with Akt Inhibitor VII resulted in inhibition of NGF-induced F-actin patch formation and filopodia formation along the axons. These data indicate that NGF-induced filopodia formation is a result of an increase in the frequency of F-actin patch formation. NGF acts through the PI3K-Akt pathway to increase the frequency of F-actin patch formation. Future studies will further investigate the mechanisms by which NGF regulates the F-actin patches responsible for filopodia sprouting.

2581/B290

Oxidative Stress Results in Defective Neuronal Transport Following Cytoskeletal Destabilization.

G. Dave, S. B. Shah; Bioengineering, University of Maryland, College Park, College Park, MD

Defective neuronal transport, characterized by a disorganized cytoskeleton and the abnormal accumulation of proteins in neurites, has been linked to the progression of several neurodegenerative conditions, including Alzheimer's disease, Amyotrophic Lateral Sclerosis, and diabetic neuropathy. We examined the role of oxidative stress, also implicated in the same set of diseases, in inducing transport defects. Photoactivation of a variety of fluorescent dyes induces the generation of reactive oxygen species (ROS). Using time-lapse fluorescence and DIC imaging, we observed the formation of swellings, which contained accumulated organelles and cytoplasmic material, in the vicinity of photoactivated fluorescent beads deposited on neuronal projections. We also measured changes in neurite geometry during swelling formation, suggesting cytoskeletal reorganization. We tested the roles of actin and microtubule networks in regulating cytoskeletal stability in response to ROS by performing similar experiments in the presence of drugs that modulate polymerization rates of actin and tubulin. Stabilization of actin with jasplakinolide or destabilization of microtubules with nocodazole accelerated swelling formation. Inhibition of actin polymerization with latrunculin slowed the rate of swelling in a majority, but not all, neurites, possibly due to differences in the integrity of pre-existing actin networks. These data suggest that neurite swellings result from local disruptions in the balance between tension generated by cortical actin and myosin, which exist in the neurite periphery, and tension borne by microtubules, which localize to the neurite core. Stabilization of microtubules with taxol did not impact swelling kinetics, suggesting that microtubules may be decoupled from the actin scaffold, but not depolymerized, during swelling. Neurite swellings are distinct from membrane blebs, which formed in the vicinity of photoactivated beads deposited on neuronal cell bodies. Experiments with jasplakinolide and latrunculin revealed that like swellings, blebs are generated through actin-myosin activity. Unlike swellings, blebs excluded larger cytoplasmic components, suggesting alternate pathways of instability.

2582/B291

Roles of Disrupted-In-Schizophrenia 1 (DISC1)-Interacting Protein Girdin in Postnatal Development of the Dentate Gyrus.

A. Enomoto[1,2], N. Asai[1], S. Taya[3], D. Tsuboi[3], K. Kuroda[3], K. Kaibuchi[3], M. Takahashi[1]; [1]Department of Pathology, Nagoya University Graduate School of Medicine, Nagoya, Japan, [2]Institute for Advanced Research, Nagoya University, Nagoya, Japan, [3]Department of Cell Pharmacology, Nagoya University Graduate School of Medicine, Nagoya, Japan

Disrupted-In-Schizophrenia 1 (DISC1) is a susceptibility gene for major psychiatric disorders, which regulates neuronal migration and differentiation during the development of mammalian brain. Recently, roles of DISC1 in the neurogenesis in the subgranular zone (SGZ) of the dentate gyrus (DG), which occurs postnatally and throughout adulthood, have been emerged (Cell 130, 1146-158, 2007). However, it remained to be determined how DISC1 and its interacting proteins govern the migration, positioning, and differentiation of dentate granule cells, the crucial processes for the neurogenesis. Here we report that DISC1 interacts with an actin-binding protein Girdin (Dev. Cell 9, 389-402, 2005; Nat. Cell Biol. 10, 329-337, 2008), which was originally identified as a substrate of the serine/threonine kinase Akt/PKB, to regulate axonal development in cultured hippocampal neurons. The localization of Girdin to the growth cones was impaired in DISC1-depleted neurons, suggesting that the role of DISC1 is to either stabilize and/or anchor Girdin at the growth cones by serving as a scaffold protein. Dentate granule cells in Girdin-deficient neonatal mice lack sprouting of axons (mossy fibers) in the cornu ammonis 3 (CA3) region of the hippocampus. The function of Girdin in the DG development is not exclusive to axonal development; its deficiency and RNA interference (RNAi)-mediated knock down lead to the overextended migration and mispositioning of the dentate granule cells that resulted in

profound cytoarchitectural disorganization of the DG. These findings identify Girdin as an important intrinsic factor for the postnatal development of the DG, as well as provide insights into the critical role of the DISC1 interactome in postnatal neurogenesis in the DG.

2583/B292

Pharmacogenetic Analysis Reveals a Post-Developmental Role for Cytoskeletal Regulators in Synaptic Transmission in C. Elegans Model.

K. P. Berry[1], S. Lee[1], B. B. Kautu[1], C. Locke[1,2], G. Caldwell[1], K. A. Caldwell[1]; [1]Biological Sciences, The University of Alabama, Tuscaloosa, AL, [2]Physiology, University of California, San Francisco, San Francisco, CA

Maintenance of proper neuronal activity relies upon a delicate balance between excitatory and inhibitory neurotransmission. Imbalances within this system result in erratic neuronal communication manifesting itself as seizures or epilepsy, a disorder that affects ~2% of the world's population. The vast majority of epilepsies have no clear cause. Thus, the identification of novel susceptibility factors is vital for understanding this disorder. Among other factors, the nerve cell cytoskeleton is essential for regulation of intrinsic neuronal activity. For example, neuronal migration defects are associated with microtubule regulators, such as LIS1 and dynein, as well as actin regulators, including Rac GTPases and integrins, and have been thought to underlie epileptic seizures in patients with cortical malformations. However, it is plausible that post-developmental functions for these cytoskeletal regulators could be masked by developmental anomalies. Accordingly, our previous results have illuminated the presence of functional and absence of developmental roles for *C. elegans* orthologs of LIS1 and dynein in GABAergic vesicle transport. Here we report that *C. elegans* with function-altering mutations in canonical Rac GTPase signaling pathway members, like *lis-1* and GABA mutants, demonstrated a robust behavioral response to a GABA receptor antagonist, pentylenetetrazole (PTZ). These mutants also exhibited hypersensitivity to an acetylcholinesterase inhibitor, aldicarb, uncovering deficiencies in inhibitory neurotransmission. RNAi against hypomorphs revealed synergistic genetic interactions between the dynein motor complex and some, but not all, members of Rac signaling pathways. These genetic interactions are consistent with putative Rac-dependent interactions between actin and microtubule networks suggesting that some cytoskeletal regulators cooperate to govern neuronal synchrony through dynein-mediated GABAergic vesicle transport in *C. elegans*. Through the combined use of pharmacological and genetic modifiers, we have established an experimental paradigm for evaluating factors influencing neuronal activity in the nematode model system that enables the rapid evaluation of prospective genetic contributors to epilepsy.

2584/B293

Nerve Growth Factor Induced Neuronal Differentiation of PC12 Cells Promotes the Interaction of βγ Subunits of Heterotrimeric G Proteins with Microtubules and Actin Filaments.

J. A. Sierra-Fonseca, O. Najera, S. Das, S. Roychowdhury; Biological Sciences, University of Texas at El Paso, El Paso, TX

Neurite outgrowth is a complex and finely regulated process that requires tight coordination between assembly/disassembly of microtubules and actin filaments and is regulated by G-protein coupled receptors in neurons. We have shown earlier that βγ subunits of G proteins are involved in promoting microtubule assembly in cultured NIH3T3 and PC12 cells. The present study was undertaken to determine if Gβγ is involved in neuronal differentiation by modulating assembly and organization of cytoskeleton. PC12 cells were used to conduct the study because they respond to nerve growth factor (NGF) with growth arrest and exhibit a typical phenotype of neuronal cells sending out neurites. PC12 cells were treated with NGF over the course of three days, and the role of Gβγ was evaluated by subcellular fractionation, co-immuno-precipitation, and confocal microscopy. We found that in cultured PC12 cells Gβγ interacts with actin filaments in addition to microtubules and this interaction was not affected by microtubule depolymerizing drug nocodazole, suggesting that the interaction of Gβγ with actin filaments is microtubule-independent. In addition, Gβγ was found to interact with AGS3, a neuron specific protein identified as receptor-independent activator of G protein-signaling. We found that microtubule assembly was increased by ~50% in NGF-treated cells. A ~200% increase in tubulin-Gβγ interaction was observed in NGF-treated cells, as demonstrated by co-immuno-precipitation analysis. In addition, Gβγ interaction with actin filaments was significantly increased in NGF-treated cells. Confocal microscopy revealed that in NGF-treated cells Gβγ was localized mainly in cellular processes in which they appear to colocalize with AGS3 and tubulin-containing structure. Our result suggests that βγ subunits of G proteins, through its interaction with AGS3, might be involved in neuronal differentiation by regulating assembly and organization of microtubules and actin filaments. This may provide a mechanism by which G protein signaling is involved in neurite outgrowth. Supported by 5G112RR08124-09 (RCMI, NIH) to the University of Texas at El Paso.

2585/B294

Rala and the Exocyst Complex Regulate Neuronal Polarity.

G. Lalli; Wolfson Centre for Age-Related Diseases, King's College London, London, United Kingdom

The cell ability to acquire a highly polarized morphology is crucial for complex biological activities, such as the organization of the nervous system. Neurons are among the best examples of highly differentiated and polarized cell types, usually extending a long thin axon designed to propagate signals, and several shorter and thicker dendrites destined to receive signal inputs. Neuronal polarization requires a tight coordination between cytoskeletal regulation and directed membrane traffic. Recent studies have uncovered a role for Ral, a Ras-like GTPase, in a variety of cellular processes, such as regulation of cell morphology, vesicle sorting and neurosecretion. In addition, the identification of the exocyst complex as a downstream effector of active Ral indicates that this GTPase could act as a regulator of polarized membrane delivery. I have previously shown that Ral regulates neurite branching in distinct neuronal cell types downstream of integrin signalling. Here, I report that the Ral isoform RalA is also involved in neuronal polarization. In developing cortical neurons both RalA depletion and alteration of RalA activity cause axon loss. Importantly, neuronal polarization is not affected in cells expressing an active RalA mutant unable to interact with the exocyst, a hetero-octameric protein complex involved in polarized membrane delivery that appears to be the only Ral effector involved in this process. Consistent with this result, knocking down expression of the exocyst subunits Sec6, Sec8 and Exo84 also leads to unpolarized neurons, which, like RalA-depleted cells, are unable to properly localize PAR-3, a crucial polarity regulator. In addition, a gradual assembly of the exocyst complex is observed at early stages of neuronal polarization and is promoted in conditions

favoring axon specification. I propose that spatial activation of Ral and local regulation of the exocyst complex contribute to the proper localization of the PAR complex essential for the establishment of neuronal polarity.

2586/B295

Promoting Axon Branching by CRMP-2 via Increasing Microtubule Assembly Dynamics.

C. Xia[1], Z. Zhao[1], Z. Wang[1], L. Ma[1,2]; [1]Zilkha Neurogenetic Institute, University of Southern California, Los Angeles, CA, [2]Department of Cell and Neurobiology, University of Southern California, Los Angeles, CA

Axonal branches are integral parts of neural circuits, and their development depends on the microtubule cytoskeleton. Although microtubule translocation has been proposed to play a major role in branch formation, the contribution of microtubule assembly dynamics is less well understood. Here, we report the use of rodent embryonic neurons from the dorsal root ganglion (DRG) as a model to probe this question and the underlying regulatory mechanism. First, in the primary culture of DRG sensory neurons, we show that dynamic microtubule assembly is required for branch formation, as low concentrations of the microtubule depolymerization drug nocodazole that inhibits microtubule dynamics can block branch formation. Second, to complement the pharmacological study, we have tested CRMP-2, a signaling protein that has been shown to bind to tubulin heterodimers and promote microtubule assembly in vitro. We show that CRMP-2 overexpression in DRG neurons enhances axonal branch formation. This activity depends on the phosphorylation of T514 in CRMP-2, as mutations at this site perturb the branch promoting activity of the protein. In addition, T514 phosphorylation can be regulated by several signaling pathways that are known to stimulate axon branching. Finally, to provide further evidence to support the role of microtubule assembly in axon branching, we have analyzed microtubule dynamics in live cells by co-expressing CRMP-2 with an EGFP-tagged microtubule plus-end binding protein EB3. We show that overexpressing either the wild type or the non-phosphorylated mutant (T514A) of CRMP-2, but not the phosphorylated mutant (T514D), leads to an increased microtubule polymerization rate and a decreased catastrophe frequency, and low concentrations of nocodazole can block CRMP-2-dependent axon branching in DRG neurons. Taken together, our study demonstrates a critical role of microtubule assembly dynamics in axon branching and links this important cytoskeletal regulation to the control of CRMP-2 phosphorylation.

2587/B296

Identification of Katanin P60 Interacting Proteins.

D. Esen Ozel[1], G. Cesur[1], P. Baas[2], A. Karabay[1]; [1]Molecular Biology Genetics and Biotechnology, Istanbul Technical University, Istanbul, Turkey, [2]Neurobiology and Anatomy, Drexel University, Philadelphia, PA

Katanin has important functions in cytoskeletal dynamics through its microtubule severing activity. Long microtubules are stationary, whereas short microtubule fragments severed via katanin achieve motility and could contribute into cytoskeletal organizations. These short and motile microtubules are especially essential for neuronal branching. Katanin protein consists of two domains; p60 catalytic domain and p80 regulatory domain. P80 directs p60 to the centrosomes where p60 forms hexameric structure and severs microtubule by ATP hydrolysis. Nevertheless, there is no identified interaction of katanin with any protein except p80 and tubulin. Thus, the purpose of this study was to identify katanin p60 interacting proteins in brain cells. In this study, Yeast Two Hybrid System, which is one of the best method for detection of novel protein interactions from cDNA pool, was chosen for identification of new katanin p60 interacting proteins. "Clontech Matchmaker Yeast Two Hybrid" kit was used for this study and new interacting proteins were selected from fetal human brain cDNA library. Katanin p60 gene was fused with DNA binding domain of transcription factor of reporter gene and cDNA library was fused with activation domain of the same transcription factor. Each fusion construct was inserted into different mating types of S. cerevisiae and strains were mated. Following mating, diploid yeast cells were selected through reporter gene activation. 22 new protein interactions were identified and confirmed. Katanin is a critical protein in microtubule dynamics and has roles in many important cellular activities. Therefore, elucidation of katanin p60 interacting proteins, revealing possible diverse roles of katanin in neurons through discovered interactions will provide significant information.

2588/B297

FGF (Fibroblast Growth Factor) Enhances Axonal Branching by Stimulating Both Katanin-based and Spastin-based Modes of Microtubule Severing.

L. Qiang, W. Yu, J. Solowska, P. Baas; Drexel University College of Medicine, Philadelphia, PA

Complex axonal arbors arise via the generation of collateral branches along the length of the parent axon. Formation of collateral branches depends on the severing of long microtubules within the parent axon to create focal enrichments of very short microtubules. These short microtubules are thought to participate in branch formation by rapidly moving into the newly forming branches. This model, termed "cut and run," predicts that growth factors such as FGF (fibroblast growth factor) that stimulate axonal branch formation do so in part by inducing changes in the severing of microtubules. Neurons express two different microtubule-severing proteins, namely katanin and spastin. Katanin is more abundant in the axon than spastin, but katanin's ability to sever microtubules is dampened by the binding of tau to the microtubules. We have proposed that axonal branches can form either by local detachment of tau from the microtubules, thus permitting katanin to access the microtubules, or by the accumulation of spastin at the branch site. Here, we report that cultures of rat hippocampal neurons undergo notable elevations in the levels of both katanin and spastin as the axon undergoes increased branching in the presence of FGF. Tau levels are the same whether or not FGF is present, but the phosphorylation of tau notably increases in the presence of FGF in a manner consistent with diminished binding to microtubules. When cultures are depleted of spastin, the frequency of branching is notably decreased, but application of FGF increases the levels of branching by the same proportion as when spastin is present. Depletion of spastin decreases the frequency of microtubule transport by roughly 30%, but the frequency of transport is partially restored when FGF is added. Taken together, these results provide strong support for the "cut and run" model for microtubule behaviors during axonal branching, and indicate that FGF stimulates both the spastin-based and the katanin-based modes of branch formation.

2589/B298

The Role of Katanin in the Drosophila Nervous System.

L. Pang, N. Sherwood; Biology, Duke University, Durham, NC

Microtubule severing proteins bind to and cut microtubules in the internal, more stable, region of the polymer. The first microtubule severing protein discovered, Katanin, functions as a hexamer formed from monomers consisting of two subunits, Katanin-60 (Kat60), the catalytic subunit, and Katanin-80 (Kat80), the regulatory subunit. Kat60 is required for normal axon extension in cultured mouse neurons, while mutations in the only other known microtubule severing protein, Spastin, cause a progressive neurodegenerative disease in humans. To determine the role of Katanin in the nervous system, the loss of function phenotype of *kat60* in *Drosophila* was characterized. Synaptic bouton morphology at the larval neuromuscular junction is affected by a loss of Kat60. Wild-type boutons appear large and round, spaced at regular intervals in linear arrays, while *kat60* null boutons are small and clustered. This phenotype is reminiscent of *spastin* mutants, where boutons are not only smaller and clumped, but increased in number. These similarities in mutant synaptic morphology may indicate overlapping functions between Kat60 and Spastin. Behavior studies revealed potential defects in nervous system function. The first of these studies, the touch-insensitivity test, is an assay for general mechanosensory defects. *kat60* nulls show touch responses indistinguishable from wild-type animals. A second test, specific for Class IV multi-dendritic (MD) neurons, assays the larval nociceptive (pain sensation) response. Wild-type larvae consistently roll only once after a mechanical stimulus is applied. In contrast, *kat60* nulls show a right shift in the distribution of rolling responses, the majority usually rolling more than once with 20% rolling 6 times. Another test, for thermal nociception, assays the activity of a different set of sensory neurons. *kat60* nulls show no difference in response compared to wild-type. Together, these behavioral tests demonstrate that *kat60* nulls are hypersensitive in their response to mechanical nociception, but not light-touch or thermal nociception, suggesting that a subset of sensory neurons are affected by *kat60* mutation.

2590/B299

Microtubule Severing, Transport and Sliding during Neuronal Migration.

A. Falnikar, P. Baas; Dept. of Neurobiology and Anatomy, Drexel University, Philadelphia, PA

Mammalian brain development involves migration of newly born neurons from their birthplace to new locales. Defects in neuronal migration often arise from mutations in genes encoding microtubule subunits or proteins related to microtubules. In the prevailing model, microtubules of a migrating neuron are envisioned as long cables with their minus ends bound to the centrosome, which itself is located between the leading process and the nucleus. Long microtubules are believed to extend forward from the centrosome into the leading process, and backward forming a cage around the nucleus. The microtubules are thought to act as cables to transmit forces from the leading edge to the centrosome and from the centrosome to the nucleus. Here we sought to investigate the merits of a very different model. In particular, we hypothesize that microtubules in a migrating neuron exist in a variety of lengths, with most of the microtubules not bound to the centrosome. In our model, the unbound microtubules undergo a variety of force-dependent behaviors such as transport if they are very short and sliding relative to each other if they are somewhat longer. In cultured migratory neurons with fluorescently labeled microtubules, we observed the rapid movement of short microtubules in the leading process, thus confirming the existence of unbound microtubules. Inhibition of katanin, a potent microtubule-severing enzyme, markedly altered the morphology of the migratory neurons and completely stopped their movement. Inhibition of kinesin-5, a molecular motor protein that acts as a "brake" on microtubule transport and sliding in the axon, caused the neurons to migrate faster but with less directional preference. Taken together, these results refute a model in which most or all microtubules are attached to the centrosome, and support a model whereby the severing, transport, and sliding of microtubules are critical factors in neuronal migration.

Dynein II (2591 – 2608)

2591/B300

The Dynein Light Chain of Budding Yeast Interacts with Dynein Intermediate Chain to Position the Mitotic Spindle*.

M. Brereton[1], A. Roberts-Siglin[2], J. Moore[1], D. Bugbee[3], N. Adames[3], J. A. Cooper[2], J. C. Williams[1,4]; [1]Cell Biology, Washington University, St. Louis, MO, [2]Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA, [3]Biological Sciences, University of Alberta, Edmonton, AB, Canada, [4]Molecular Medicine, Beckman Research Institute, Duarte, CA

Cytoplasmic dynein is a large multisubunit complex involved in the retrograde transport of vesicles and organelles along microtubules as well as checkpoint signaling and chromosome separation. Of these subunits, the dynein light chains (LCs), LC8 and TcTex1, have been shown to bind to multiple protein targets in addition to the dynein intermediate chain (IC), suggesting that they may function to bridge cargo to the motor for retrograde transport. Our structural and thermodynamic studies of the LCs however, show that the multiple protein targets also bind the same site on the LCs as the dynein IC. These data, taken together with recent functional data, suggest that the LCs regulate the activity of their targets, perhaps through an allosteric mechanism. To test this possibility, we asked whether LC8's interaction with the IC is responsible for its ability to position the nucleus in budding yeast. To this end, we identified the molecular basis of the interaction and constructed point mutations in the dynein IC (pac11 in yeast) that specifically impaired the interaction. These point mutations produced a defect in positioning of the spindle that was identical to that of null mutations that completely removed the LC8 (Dyn2 in yeast) or the intermediate chain of the dynein complex. These results provide the first functional evidence that the LC occupancy of the dynein motor complex is required for a major physiological process. * Melissa Brereton & Amanda Roberts-Siglin are co-first authors

2592/B301

The P25/P27 Subunits of Dynactin Associate with a Juxta-Nuclear Membrane Compartment.

B. R. Scipioni[1], T. Yeh[1], N. Quintyne[1, 2], T. Schroer[1]; [1]Biology, Johns Hopkins University, Baltimore, MD, [2]Biology, Florida Atlantic University, Jupiter, FL

Dynactin is a multi-subunit protein complex that serves as an adaptor between microtubule-based motors and their cargo. We have biochemically shown that the p25 and p27 subunits of dynactin are the only two dynactin-specific subunits to exist in a sizeable pool outside of dynactin. p25 and p27, which form a heterodimer, are components of dynactin's pointed end complex, the domain of dynactin responsible for cargo binding and selection. Although dynactin is conserved from yeast to humans, the p25 and p27 subunits have not been identified as components of the yeast dynactin complex. Unlike dynactin in multicellular fungi and metazoans, organisms which use dynactin for membrane-related and mitotic events, yeast dynactin is only required for proper positioning of the mitotic spindle. That yeast dynactin does not appear to contain a p25/p27 homolog suggests that these subunits contribute in some way to membrane associated dynactin functions. Immunolocalization of endogenous p27 reveals a Golgi-like juxta-nuclear cluster in both Cos7 and HeLa cells. This pattern has not been observed through immunolocalization of any other dynactin subunit for which reagents are available, and appears to be cell cycle dependent. This novel "compartment" is disrupted by nocodazole, BFA, and a 15°C temperature shift, conventional treatments used to disrupt internal membrane compartments. We conclude that the p25/p27 heterodimer associates with an internal membrane compartment in a cell cycle dependent manner. These findings may provide important new clues regarding dynactin function and regulation.

2593/B302

Dynein Light Intermediate Chain: an Essential Subunit That Contributes to Spindle Checkpoint Inactivation.

Y. He[1], S. Mische[1], L. Ma[1], M. Li[1], I. McLeod[2], J. Thompson[2], M. Serr[1], J. R. Yates[2], T. Hays[1]; [1]Dept. of GCD, University of Minnesota, Minneapolis, MN, [2]Dept. of Cell Biology, Scripps Research Institute, La Jolla, CA

The dynein light intermediate chain (LIC) is a subunit unique to the cytoplasmic form of dynein, but how it contributes to dynein function is not fully understood. Previous work has established that the LIC homodimer binds directly to the dynein heavy chain, and may mediate dynein's attachment to centrosomes and other cargoes. Here we report our characterization of the LIC in Drosophila. Unlike vertebrates, in which two Lic genes encode multiple subunit isoforms, the Drosophila LIC is encoded by a single gene. We determined that the single LIC polypeptide is phosphorylated, and that different phosphoisoforms can assemble into the dynein motor complex. Our mutational analyses demonstrate that, similar to other dynein subunits, the Drosophila LIC is required for zygotic development, germline specification of the oocyte, and mitotic cell division. We show that RNAi depletion of LIC in Drosophila S2 cells does not block the recruitment of a dynein complex to kinetochores, but does delay inactivation of Mad2 signaling and mitotic progression. Ongoing experiments are investigating whether phosphorylation of the LIC regulates dynein activity and the spindle checkpoint. Support: NIH RO1-GM44757 to TSH and NIH P41 RR011823 to JRY

2594/B303

The p27 Subunit of Dynactin Governs Entry into Mitosis.

T. Yeh[1], B. R. Scipioni[1], F. Cheong[1], N. Quintyne[1, 2], T. Schroer[1]; [1]Biology, Johns Hopkins University, Baltimore, MD, [2]Biology, Florida Atlantic University, Jupiter, FL

Dynactin functions as an activator and cargo adaptor for the cytoplasmic dynein motor in many types of microtubule minus-end directed transport. Previous studies have shown that dynactin complex contains three major structural domains: the sidearm-shoulder, the Arp1 minifilament, and the pointed end complex. p27 is one of the subunits in the pointed end complex, but its cellular function remains enigmatic. Here we report a novel cell cycle dependent behavior for dynactin p27 and explore its role in cell cycle progression. p27 is phosphorylated in G2 and M phase, suggesting that its function is regulated by the mitotic kinase(s). Immunolocalization revealed that p27 distribution dramatically changes through G1 to G2 phase. During mitosis, p27 labeling was undetectable at spindle poles or kinectochores like other dynactin subunits. Disruption of dynactin has been shown to cause an increase in mitotic index and pseudoprometaphase arrest due to spindle defects. In contrast, p27 knockdown yields a decrease in mitotic index and has no effect on spindle structure, suggesting that p27 is dispensable for dynactin's function in spindle formation. Furthermore, overexpression and knockdown of p27 cause delayed and premature entry into mitosis, respectively. Our findings suggest that dynactin may act as a novel regulator for the checkpoint of mitotic entry.

2595/B304

Anaphase Dynein Activation Controlled by Dynactin Recruitment to Astral Microtubules.

J. Woodruff, D. Drubin, G. Barnes; UC Berkeley, Berkeley, CA

Dynein is a minus-end-directed microtubule motor important for mitotic spindle positioning. In budding yeast, dynein activity is restricted to anaphase when the nucleus must enter the bud neck, yet the nature of this regulatory mechanism is not known. Here, the spindle protein She1 is identified as a novel regulator of dynein activity. In *she1Δ* cells, dynein is prematurely activated and pulls the spindle back and forth across the bud neck before anaphase. Additionally, without She1, dynein remains active after anaphase and pulls the spindle-organizing centers around the cell cortex. Finally, we found that dynactin, a cofactor essential for dynein motor function, is a dynamic complex whose recruitment to astral microtubules is cell cycle stage-dependent, and that She1 precludes association of the complete dynactin complex with microtubules until anaphase. Therefore, we propose that She1 restricts dynein activity to anaphase by inhibiting the recruitment of the complete dynactin complex to astral microtubules, thereby preventing productive association of dynein with dynactin.

2596/B305

Mechanisms of Dynein Targeting during Mitosis.

C. G. Lazar, M. A. Morrissey, J. Bader, J. M. Kasuboski, K. Vaughan; Department of Biological Sciences, University of Notre Dame, Notre Dame, IN

Cytoplasmic dynein is an essential microtubule motor protein that plays important roles at the cell cortex, spindle poles and kinetochores during mitosis. The mechanisms of targeting to these distinct loci remain unclear. Using mass spectrometry of mitotic dynein, we mapped two novel phosphorylation sites in the dynein intermediate chains (ICs) and prepared antibodies to phosphorylated peptides at each site. Antibodies against phosphorylation at T89 in the ICs (PT89) label kinetochores from nuclear envelope breakdown to metaphase. Loss of PT89 dynein labeling on aligned chromosomes correlated with robust labeling of kinetochore fibers and spindle poles with antibodies against

dephosphorylated dynein ICs. Binding of PT89 dynein to kinetochores was dependent on the mitotic checkpoint protein zw10 rather than dynactin and was affected by shRNA against zw10 as well as inhibition of the mitotic kinase Aurora B. The latter did not influence PT89 phosphorylation directly but rather the assembly of the RZZ (rod, zw10, zwilch) platform for dynein recruitment. Antibodies against phosphorylation at Y130 in the ICs (PY130) labeled spindle poles rather than kinetochores and persisted throughout mitosis. Inhibition of dynein recruitment to kinetochores or disruption of metaphase-induced poleward streaming from kinetochores did not affect PY130 labeling, suggesting a functional difference from PT89 dynein at kinetochores. Transfection of GFP-tagged NuMa revealed that PY130 dynein and NuMa colocalize at spindle poles. Transfection of a dominant-negative NuMa construct disrupted spindle poles and redistributed PY130 dynein to a soluble pool. These findings suggest that phosphorylation of the dynein ICs generates two different populations of dynein that localize to kinetochores or spindle poles. Site-specific disruption suggests that kinetochore and spindle pole dynein play distinct roles during mitosis.

2597/B306

Cytoplasmic Dynein Is Required for Proper Positioning of the Meiotic Spindle in C. Elegans.

M. L. Ellefson, F. McNally; Molecular and Cellular Biology, University of California Davis, Davis, CA

Asymmetric spindle positioning is a conserved component of female meiosis in animals. Proper spindle positioning is required for reduction in chromosome number. Failure to execute reduction of chromosome number results in polyploid embryos after fertilization, commonly leading to embryonic lethality. In human and *C. elegans* female meiosis, the spindle is juxtaposed perpendicular to the cortex at anaphase allowing for efficient segregation of extra chromosomes into polar bodies. Both human and *C. elegans* meiotic spindles are acentriolar, thus it is likely that the mechanisms driving spindle positioning are similar. During *C. elegans* wild-type meiosis, the spindle is assembled at a distance from the cortex, then translocates to the cortex in an orientation parallel to the cortex. This parallel translocation depends on kinesin-1 (UNC-116). Following APC activation, the wild-type spindle undergoes a distinct rotation to a perpendicular orientation. In the absence of UNC-116 early parallel translocation fails. However, after APC activation the spindle moves to the cortex with one-pole leading, resulting in a perpendicular orientaton at the cortex by late anaphase. These observations led to a model in which the same APC-activated motor protein mediates rotation in wild-type embryos and late translocation in *unc-116* mutant embryos. Using RNAi, we have identified cytoplasmic dynein, DHC-1, as the motor responsible for these late, APC-dependent movements. Depletion of DHC-1 in wild-type and *unc-116* mutant backgrounds results in failure of spindle rotation and late spindle translocation, respectively. A combination of live imaging of transgenic animals and immunofluorescence of fixed animals has allowed us to observe the localization pattern of cytoplasmic dynein during meiosis. During metaphase. dynein is localized faintly on the entire meiotic spindle. After APC activation, just prior to rotation, dynein accumulates on the spindle poles and remains on the poles through completion of anaphase. Because dynein relocalization to microtubule minus ends at spindle poles occurs at the same time as spindle rotation, we hypothesize that dynein motility is activated by proteolysis of an APC substrate.

2598/B307

Self-Organization of Dynein Motors Generates Meiotic Nuclear Oscillations.

S. Vogel[1], N. Pavin[2], N. Maghelli[1], F. Julicher[2], I. Tolic-Norrelykke[1]; [1]MPI-CBG, Dresden, Germany, [2]MPI-PKS, Dresden, Germany

Molecular motors position organelles in eukaryotic cells. A central question is how a multitude of motors organize their behavior into a concerted movement. Here we report a mechanism of meiotic nuclear oscillations in *Schizosaccharomyces pombe* based on collective behavior of dynein motors acting on oppositely oriented dynamic microtubules. By combining quantitative live cell imaging and laser ablation with a new theoretical model, we show that dynein dynamically redistributes in the cell in response to load forces, resulting in more dynein attached to the leading than to the trailing microtubules. The load-dependent redistribution introduces the force asymmetry into the tug-of-war between motors exerting forces in opposite directions, thereby generating oscillations. Our work provides the first direct *In Vivo* observation of self-organized dynamic dynein distributions, which, due to the intrinsic motor properties, generate regular large-scale movements in the cell.

2599/B308

Loss of Huntingtin Leads to Defects in Endocytosis.

J. Caviston, E. Holzbaur; Physiology, University of Pennsylvania School of Medicine, Philadelphia, PA

Huntingtin (Htt), a vesicle-associated protein found in both neuronal and non-neuronal cells, binds to cytoplasmic dynein and facilitates dynein-mediated vesicular transport along the microtubule. Mutant Htt contains an expansion of the polyglutamine repeat region that causes the neurodegeneration seen in Huntington's Disease, however the normal function of the protein is not fully understood. Dynein and its activator dynactin are thought to mediate endosomal motility, as disruption in dynein/dynactin function results in endocytosis defects. Here we show that Htt RNAi cells also demonstrate a defect in endocytosis. We utilized fluorescently labeled transferrin to follow the trafficking of the transferrin receptor (TfR), which is constitutively recycled. In Htt RNAi cells, TfR was trafficked to endocytic compartments that failed to localize near the cell center and instead remained in small clusters more widely distributed within the cytoplasm. Mislocalization was accompanied by a delay in uptake of TfR from the plasma membrane relative to control cells. Defects in endosomal trafficking were not limited to the early endosomes, as lysosomes were also widely dispersed throughout the cytoplasm, in contrast to the normal juxtanuclear positioning found in control cells. For comparison, we assessed endocytosis in dynactin knockdown (p150Glued RNAi) cells. These cells demonstrated a more pronounced defect in TfR trafficking; the mislocalization of lysosomes was also more dramatic in dynactin knockdown cells. These results support a model in which Htt functions as a vesicle-associated scaffold that enhances interactions between endocytic compartments and dynein/dynactin. Htt is also known to interact with kinesin through Htt-associated protein 1 (HAP1), and thus may coordinate bidirectional microtubule-based motility of vesicular cargo in the cell, including both early and late endosomes. Supported by NIH GM48661.

2600/B309

Spinocerebellar Ataxia Type 5 Mutations Cause Synaptogenesis and Axonal Transport Defects in Drosophila.

D. Rorenzo[1,2], M. Li[1], L. Ranum[1,2], T. Hays[1]; [1]Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, [2]Institute of Human Genetics, University of Minnesota, Minneapolis, MN

Spinocerebellar ataxia type 5 (SCA5) is a progressive autosomal dominant neurodegenerative disease characterized by incoordination, slurred speech and eye movement abnormalities. MRI and autopsy findings show cerebellar cortical atrophy and profound Purkinje cell loss. SCA5 is caused by mutations in beta-III spectrin (SPTBN2), a cytoskeletal protein highly expressed in Purkinje cells. Beta-III spectrin has been previously implicated in the stabilization of specialized synaptic proteins including the Purkinje cell specific glutamate transporter, EAAT4, at the plasma membrane as well as in the transport of vesicles through its interaction with the Arp1 subunit of dynactin. Whether these attributes of beta-III spectrin contribute to the neurodegenerative phenotype remains unclear. To investigate the molecular basis of SCA5 and test the hypothesis that SCA5 pathogenesis may result from defects in the machinery of intracellular transport, we have generated a Drosophila model of the disease. Pathological SCA5 mutations expressed in the context of endogenous fly beta spectrin using the GAL4/UAS system cause larval posterior paralysis, focal axonal swellings filled with vesicle-associated cargoes and motor proteins, and significant reductions in the anterograde and retrograde run velocities of synaptic vesicles. Furthermore, expression of mutant but not wild type fly beta spectrin in larval motor neurons causes a considerable terminal atrophy ($p=<0.0001$) characterized by reduced branching and bouton number at neuromuscular junctions. These observations suggest a role for beta spectrin in the transport of structural and signaling components required for synaptogenesis and neuronal function. Additionally, a candidate gene based modifier screen has detected genetic interaction between mutant beta-spectrin and components of the dynein/dynactin transport machinery in flies. We are now conducting a genome-wide genetic screen to further elucidate the molecular underpinnings of SCA5.

2601/B310

Alterations in Retrograde Signaling Lead to Neurodegeneration.

E. Perlson[1], J. Ross[1], G. Jeong[2], R. Dixit[1], K. Wallace[1], R. Kalb[2], E. Holzbaur[1]; [1]Department of Physiology, University of Pennsylvania, Philadelphia, PA, [2]Children's Hospital of Philadelphia, Philadelphia, PA

Retrograde axonal transport driven by dynein is vital for neuronal survival. Here, we test the hypothesis that alterations in retrograde signaling lead to neurodegeneration. Live cell imaging and in-vivo assays show an impairment of axonal transport, including a shift to slower velocities and increased pausing in the SOD1G93A model for familial ALS. Dynein function is not directly affected; instead, the coordinated motility of vesicular cargo is altered, suggesting changes in motor regulation. Using a proteomics approach to investigate the dynein-associated signaling compartment in the SOD1G93A model relative to wild-type, we observed a significant decrease in survival factors including pTrk and pErk1/2, and an increase in stress factors including pJNK, Caspase-8 and p75 cleavage fragment. Similar changes are not seen in a more slowly progressive model of neurodegeneration due to defective dynein function. Together, these data indicate that spatial changes in the cellular balance of retrograde survival/stress signals contribute significantly to neurodegenerative disease.

2602/B311

Neurodegeneration Mutations in Dynactin Impair Force Production by Dynein In Vivo.

J. Moore[1], D. Sept[2], J. Cooper[1]; [1]Department of Cell Biology and Physiology, Washington University, St. Louis, MO, [2]Department of Biomedical Engineering and Center for Computational Biology, Washington University, St. Louis, MO

Neurodegenerative disease in humans can be caused by mutations affecting the microtubule motor dynein. Dynactin is a multi-protein complex required for dynein to function. A single amino-acid change, G59S, in the p150glued subunit of dynactin can cause an ALS-like motor neuron disease in humans and mice. The molecular mechanism by which this mutation causes neuronal defects is not understood. In particular, it is not known whether the G59S substitution impairs the function of dynein. Here, we investigate the consequence of this mutation for dynein activity in budding yeast, where the sole function of dynein is to apply a pulling force to cytoplasmic microtubules from the cell cortex in order to draw the mitotic spindle across the bud neck. Using high-resolution timelapse microscopy of live cells, we show that the G59S mutation produces a highly specific phenotype related to dynein function, and the effect of the point mutation is identical to that of complete loss of the conserved CAP-Gly (cytoskeletal-associated protein glycine-rich) domain of p150glued. Our results demonstrate that the CAP-Gly domain has a critical role in the initiation and persistence of dynein-dependent movement of the mitotic spindle and nucleus, particularly during the initial movement of the nucleus through the bud neck, which involves a substantial constriction of the nuclear volume. However, we find that the CAP-Gly domain is not absolutely required for dynein activity, as the absence of this domain does not affect the sliding of detached microtubules along the cell cortex. This suggests that the need for the CAP-Gly domain depends on the load applied to the dynein motor, and CAP-Gly functionality may only be necessary when dynein needs to overcome high force thresholds in order to produce movement.

2603/B312

Transplanting the Stalk and Microtubule-Binding Domains of Chlamydomonas Axonemal Inner Arm Dynein into Dictyostelium Cytoplasmic Dynein.

K. Imamula[1], T. Kon[1], T. Yagi[2], R. Ohkura[1], K. Sutoh[1], K. Sutoh[1]; [1]Life Sciences, University of Tokyo, Tokyo, Japan, [2]Kyoto University, Kyoto, Japan

Most of Chlamydomonas inner arm dyneins such as dynein c drive rotation of sliding microtubules around their axis, while one of the Chlamydomonas inner arm dynein, dynein f, does not. This rotation may be somehow related to ciliary and flagellar movement. In order to identify which part of dynein is responsible for the rotation, we made recombinant dynein motor domains based on Dictyostelium cytoplasmic dynein, which does not drive the microtubule rotation. The coiled-coil stalk plus microtubule binding domain (MTBD) of Dictyostelium dynein were replaced by those of Chlamydomonas dynein. The resulting chimera dyneins drove the microtubule rotation only when the stalk plus MTBD from dynein which can drive the rotation was used, suggesting that the stalk plus MTBD are responsible for the rotation. To further dissect the location responsible for the rotation, the stalk coiled-coil and MTBD from Chlamydomonas dynein were separately transplanted into Dictyostelium dynein. The result showed that the stalk coiled-coil, not MTBD, is responsible for the rotation. The stalk coiled-coil of Chlamydomonas dynein with rotation activity typically contains a pro residue, while dynein f does not, suggesting that

this pro may be responsible for the rotation. To test this prediction, the pro residue was mutated to ala residue. This pro/ala mutation did not alter the rotation, indicating the pro residue is not responsible for the rotation. In summary, our transplanting studies have shown that the stalk coiled-coil and MTBD can be replaced without affecting their functions, and that a particular structure of the stalk coiled-coil, not the single pro residue, is responsible for the rotation of sliding microtubules around their axis that is driven by most of Chlamydomonas inner arm dyneins.

2604/B313

Fine Mapping of the Dynein-Dynactin Interface.

A. Roberts-Siglin[1], S. Tan[2], M. Poenie[2], J. C. Williams[1,3]; [1]Biochemistry and Molecular Biology, Thomas Jefferson University, Philadelphia, PA, [2]Molecular Cell and Developmental Biology, University of Texas, Austin, TX, [3]Molecular Medicine, Beckman Research Institute, Duarte, CA

In conjunction with dynactin, cytoplasmic dynein participates in a number of cellular processes including retrograde transport of vesicles, distribution and maintenance of organelles and cell division. The principle subunits that mediate this interaction have been mapped to the dynein intermediate chain and the p150glued subunit of dynactin. While previous reports find residues 1 to 106 of the IC are required for p150glued binding, the IC binding site on dynactin remains unclear. Moreover, the mechanism by which the interaction is regulated remains poorly defined. Herein, we present data consisting of Ni-pulldown assays, native PAGE, and SEC chromatography to refine the interface and show that the residues 1 to 32 of the dynein IC are sufficient for binding p150glued. In addition, we show that residues 415 to 530 of p150glued can bind to the dynein IC. Using this mapping, we show by sedimentation equilibrium experiments that individual fragments are predominantly monomeric though strongly predicted to form a coiled coil entity. Admixtures of the IC and p150glued fragments, however, produce a 2:2 complex. In addition, these sedimentation experiments reveal that tetrameric complex is sensitive to salt, temperature and pH indicating that the interaction is dominated by electrostatic interactions. Taken together, we propose that the dynein-dynactin interaction proceeds through a disorder-to-order transition. We discuss the implications our findings and this model in the context of the interaction and its regulation

2605/B314

In Situ Structure of the Dynein-Microtubule-Complex in Flagella.

M. Raytchev[1], T. Heuser[1], M. E. Porter[2], D. Nicastro[1]; [1]Biology Dept., Brandeis University, Waltham, MA, [2]Dept. of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN

Dyneins are large minus-end directed microtubule (MT) motors that convert chemical energy derived from ATP hydrolysis into mechanical force[1,2]. They participate in a wide variety of cellular processes including movements of cargo along MTs, and motility of cilia and flagella[1-4]. Axonemal dyneins cause sliding motion between neighboring MT doublets, which is transformed into bending by structures that restrict sliding (e.g. nexin links)[1,2]. Dyneins bind to MTs in an ATP-sensitive manner by a small globular domain located at the tip of a long stalk that emerges between AAA-domains 4 and 5 from the motor domain[5,6]. In contrast to kinesin and myosin, the MT-binding domain is well separated from the site of ATP hydrolysis in AAA-domain 1[7]. It has been shown that the cargo-binding tail (ATP-insensitive MT binding) is important for dynein motility[7], but the detailed domain structure of the dynein-MT complex[8,9] and the mechanisms by which nucleotide hydrolysis events are regulated and transformed into movement are unknown. We therefore study the 3D structure of the dynein-MT complex in intact *Chlamydomonas* axonemes using cryo-electron tomography in combination with image processing[4]. We were able to resolve single domains and their relative position in flagellar outer dyneins in situ. In particular, we could visualize the position of the tail and C-terminal domain in relation to the motor domain. The tails seem to come in close contact with AAA 3 and AAA 4, which are thought to have regulatory function. In the future we will try to increase our resolution of dynein and visualize different states of its mechano-chemical cycle to ultimately provide a comprehensive understanding of how dyneins work at the molecular level. Supported by GM083122 and Pew Scholars Award (DN), and GM55667 (MP). References: [1] Gibbons IR (1981) J Cell Biol 91:107s. [2] King S (2003) In Molecular Motors, Chapter 2. Wiley-VCH. [3] Porter ME & Sale WS (2000) J Cell Biol 151:F37. [4] Nicastro D *et al* (2006) Science 313:944. [5] Burgess SA *et al* (2003) Nature 421:715. [6] Samso M & Koonce MP (2004) J Mol Biol 340:1059. [7] Shima T *et al* (2006) PNAS 104:17736. [8] Oda T *et al* (2007) J Cell Biol 177:243. [9] Mizuno N *et al* (2007) PNAS 104:20832.

2606/B315

Simple and Reliable Identification of Microtubule Polarity for Molecular Motor Assays.

R. Mallik, V. Soppina, A. K. Rai; Biological Sciences, Tata Institute of Fundamental Research, Mumbai, India

A new method to label minus ends of microtubules for use in in-vitro assays of molecular motor based transport is presented. This method does not require fluorescent labeling, nor the use of purified axonemes, and neither does it require antibodies to specific ends of the microtubule. The method is particularly suited for studies of bidirectional microtubule-motor driven motion of cellular organelles in reconstituted assays. Reagents required are inexpensive and common, and are either commercially available or easy to prepare in the laboratory. The reliability of this microtubule polarity labeling is demonstrated using a motility assay of native conventional Kinesin derived from goat brains. We reconstitute bidirectional molecular motor driven motion of an extract of vesicles from Dictyostelium on the labeled microtubules, and use an optical trap for preliminary estimates of force exerted by minus and plus-directed microtubule motors. The plus-end Kinesin motors attach more tenaciously to microtubules under load applied by the optical trap, while the minus-end Dynein motors detach frequently. Our method of microtubule polarity labeling allows us to differentially estimate the activity of minus and plus-directed motor complexes on endogenous vesicles, and opens an exciting avenue to probe function of specific classes of molecular motors within native motor complexes.

2607/B316

Structure and Functional Role of Dynein's Microtubule Binding Domain.

A. Carter[1], J. E. Garbarino[2], C. Cho[1], W. Shipley[2], R. D. Vale[1], I. R. Gibbons[2]; [1]Cell and Molecular Pharmacology, UCSF, San Francisco, CA, [2]Molecular and Cell Biology, UC Berkeley, Berkeley, CA

Dyneins are a family of minus-end-directed microtubule motor proteins that move various cargos within the cytoplasm and power the beating of cilia and flagella. An unusual feature of dynein is that its microtubule-binding domain (MTBD) is separated from its ring-shaped AAA+ ATPase domain by a 15 nm coiled-coil stalk. Here, we report the crystal structure of the MTBD and a portion of the coiled coil and provide functional data that the MTBD, and not the ATPase domain, specifies the direction of dynein motility. Our data suggest a model of how the MTBD, stalk and ATPase domains cooperate to drive the minus-end-directed motion of the dynein motor.

2608/B317

Force-Dependent Stepping of Cytoplasmic Dynein.

A. Gennerich, A. Carter, S. L. Reck-Peterson, R. Vale; Mol. & Cell. Pharmacology, UCSF, San Francisco, CA

Cytoplasmic dynein is a two-headed molecular motor found in eukaryotic cells that uses the energy from ATP hydrolysis to move toward the minus ends of microtubules. Cytoplasmic dynein, which is involved in a variety of motile processes such as mitotic spindle formation and the directed transport of organelles and mRNA, is composed of two identical ~500 kDa heavy chains and several associated proteins. The heavy chain contains 6 AAA domains (AAA: ATPase associated with diverse cellular activities) arranged in a ring. The first four AAA modules (AAA1-AAA4) have conserved nucleotide-binding and hydrolysis motifs. AAA domain 1 is essential for dynein motility, while the other sites (particular AAA3) may contribute important regulatory functions. In order to dissect the dynein mechanism, we used optical tweezers to analyze the force-dependent stepping behavior of native and truncated artificially dimerized yeast cytoplasmic dynein. At low loads (1 pN), dynein primarily advances by 8 nm as well as occasionally larger (12-24 nm) increments. Increasing loads (3-6 pN) frequently induce large (12-24 nm) alternating forward-backward displacements that fail to advance the motor and might be caused by a force-induced conformational change in the positioning of the two motor domains in the dynein dimer. Loads close to dynein's stall force of 7 pN decrease dynein's primary advancing step size from 8 to 4 nm, and dynein walks backward toward the microtubule plus-end at forces above 7 pN. Remarkably, in the absence of ATP, dynein steps processively along microtubules under an external load, with less force required for minus-end- than for plus-end-directed movement. This nucleotide-independent walking reveals that force alone can drive repetitive microtubule detachment-attachment cycles of dynein's motor domains. I will present a walking model for cytoplasmic dynein that accounts for the observed force- and nucleotide-dependent stepping of the two-headed motor and as well as recent microtubule unbinding force measurements of engineered single-headed dynein.

Kinesin (2609 – 2633)

2609/B318

The KCA-1 Protein Mediates Kinesin 1-Dependent Movement of Multiple Cargoes during Meiosis in C. Elegans.

J. Martin, K. McNally, F. McNally; Molecular and Cellular Biology, University of California, Davis, CA

Previously we showed that translocation of the meiotic spindle to the cortex in C. elegans requires kinesin 1 and a kinesin 1-binding protein, KCA-1. We hypothesized that KCA-1 acts as a spindle-specific 'cargo adaptor' protein for kinesin 1. In disagreement with this hypothesis, we have identified two additional cargoes that require both kinesin 1 and KCA-1 for movement. The first of these cargoes is the nucleus of the -1 oocyte. In wild-type, this nucleus migrates toward the junction with the -2 oocyte at 0.37 µm /min (n=6). The result of this migration is that the nucleus is 1.32 +/- 0.47 µm from the cortex at the time of nuclear envelope breakdown (NEBD). In contrast, no migration of nuclei was observed in worms depleted of kinesin 1 or KCA-1 by RNAi and the distance of these nuclei from the cortex at the time of NEBD was 8.43 +/- 1.67µm (n=14) and 6.56 +/- 2.18µm (n=25), respectively. The second type of novel cargo includes endosome-related yolk granules that move during "cytoplasmic streaming" in meiotic embryos. In wild-type, cytoplasmic streaming begins shortly after the embryo exits the spermatheca and continues until anaphase of meiosis II. No streaming was observed in worms depleted of either kinesin 1 (n=6) or KCA-1 (n=8). These results suggest that KCA-1 acts as a generalized activator of kinesin 1 during meiosis rather than as a cargo-specific adaptor. To test whether kinesin 1 acts directly as the motor moving each of these cargoes, we used immunofluorescence and fluorescent protein fusions to test whether kinesin 1 or KCA-1 localizes on oocyte nuclei, meiotic spindles or yolk granules. Immunofluorescence with antibodies specific for the kinesin 1 heavy chain,UNC-116, or the kinesin 1 light chain, KLC-2, revealed staining around meiotic spindles, supporting their direct involvement in meiotic spindle translocation. Additionally, mCherry-KCA-1 and GFP-KCA-1 were observed in cytoplasmic punctae within oocytes. These punctae are similar in appearance to yolk granules labeled with GFP-vitellogenin. These results support a direct role for KCA-1 and kinesin 1 in the movement of multiple cargoes during meiosis.

2610/B319

The Kinesin-1 Tail Is Not Important for Cytoplasmic Streaming in the Drosophila Oocyte.

P. Moua[1], W. Saxton[2]; [1]Biology, Indiana University, Bloomington, IN, [2]MCD Biology, University of California Santa Cruz, Santa Cruz, CA

Intracellular transport relies on complex interactions between molecular motor proteins and the actin and microtubule cytoskeletons. In *Drosophila melanogaster* the microtubule motor Kinesin-1 is responsible for driving axonal transport in the larval nervous system and cytoplasmic streaming in the oocyte. These provide us with two distinct modes of intracellular transport to investigate how kinesin might function in different transport processes. We recovered 28 kinesin-1 heavy chain (Khc) missense alleles from a series of genetic screens, 4 of which had codon changes in the globular tail region. Intra-allelic lethal complementation showed that the tail alleles were similar in severity to hypomorphic mutations in the motor domain, indicating that individual residues in the tail are essential for viability. Previous studies suggest that the Khc tail helps regulate motor domain activity and that it may interact with kinesin-1 cargoes. To investigate the role of the Khc tail in vivo, we compared the effects of three mutant tail alleles with two motor domain alleles in axonal transport and cytoplasmic streaming. All 5 alleles caused axonal swellings in motor neurons suggesting a disruption in axonal transport. Time-lapse microscopy showed severe reductions (2-8 fold) in mitochondrial flux for tail mutations and an almost complete inhibition for motor domain mutations. These

results indicate that both the tail and the motor domain are critical for organelle transport in axons. For cytoplasmic streaming assays, we tracked yolk endosomes in live oocytes to determine mean velocities. Surprisingly, while motor domain alleles reduced streaming velocities 3-10 fold, the tail alleles had no significant effects. Interestingly, both the tail and motor domain alleles caused defects in dorsalization. This indicates that although the Khc tail residues tested are not important for streaming, they are important for other kinesin-1 requiring processes in the oocyte. Our results suggest that oocytes can use kinesin-1 in a novel manner, raising interesting questions about motor regulation and how Khc interacts with the cytoplasmic components that act as impellers for fast streaming.

2611/B320

Analysis of the Localizations of GFP-Tagged Kinesins in the Filamentous Fungus Aspergillus Nidulans.

T. Akashi[1], T. Horio[2], B. Oakley[2]; [1]Molecular Mycology and Medicine, Nagoya University Graduate School of Medicine, Nagoya, Japan, [2]Molecular Genetics, Ohio State University, Columbus, OH

The fungus Aspergillus nidulans has 11 genes of the kinesin superfamily. We have found that only the kinesin encoded by bimC is essential. Deletants of the other kinesin genes were viable and only a few of the deletants exhibited minor growth defects, suggesting that most of the kinesins share overlapping functions with other molecules. We have also analysed the localization patterns of the kinesins tagged with mCherry or GFP. mCherry- and GFP-tagged kinesins generally showed the same localization patterns, but the GFP signal was stronger and revealed additional detail in some cases. We report the localization patterns of two previously uncharacterized kinesins. The class 6 kinesin (MKLP1/Kif23 group) encoded by AN3124 localized to the central spindle in anaphase/telophase and to the forming septum after mitosis. The class 10 kinesin (kid group) encoded by AN3721 accumulated briefly in the nucleus just before mitosis, loaded briefly onto the short, forming spindle and then disappeared as spindle formation proceeded. These novel localization patterns are distinctive and could provide valuable clues as to the functions of these kinesins. Supported by GM031837 from the NIH.

2612/B321

Kinesin-14A and Kinesin-14B Localization and Function in the Widespread Parasite Giardia Intestinalis.

E. Grau[1], A. Paradez[2], S. House[1], S. C. Dawson[1]; [1]Microbiology, UC Davis, Davis, CA, [2]Molecular and Cell Biology, UC Berkeley, Berkeley, CA

Giardia intestinalis is a binucleate parasitic protist with a complex microtubule cytoskeleton and twenty-four kinesins. Two kinesins are members of the C-terminal kinesin 14A and kinesin 14B families. In diverse eukaryotes kinesin-14 functions include mitosis (kinesin-14A) and organelle transport (kinesin-14B). To determine the role of kinesin-14 homologs in *Giardia*, we first localized and determined the turnover kinesin::GFP fusions using live cell imaging and FRAP. Kinesin-14A::GFP localized to both nuclei during interphase and to the spindle poles during mitosis. Kinesin-14B::GFP primarily localized to the cytoplasmic regions of the caudal and anterior axonemes. To further characterize the role of kinesin-14 homologs in *Giardia*, we overexpressed dominant negative rigor mutants and observed mitotic and flagellar defects. As in all eukaryotes, we suggest that kinesin-14A has a conserved mitotic function in *Giardia* and that the kinesin-14B homolog has a role either cargo transport along or movement of the "caudal complex" a microtubule structure enclosing the caudal axonemes.

2613/B322

Regulation of Budding Yeast Kinesin-8 by Its Tail Domain.

X. Su[1,2,3], M. Gupta[1,2,3], D. Pellman[1,2,3]; [1]Pediatric Oncology, Dana-Farber Cancer Institute, Boston, MA, [2]Howard Hughes Medical Institute, Boston, MA, [3]Harvard Medical School, Boston, MA

The spatial and temporal control of microtubule dynamics is important for cell morphogenesis, intracellular transport, and especially for mitosis. Kip3, the kinesin-8 in budding yeast, is both a microtubule plus-end-directed motor and plus-end-specific depolymerase. It is involved in processes throughout budding yeast mitosis, such as spindle positioning, chromosome segregation and spindle disassembly. Similar to conventional kinesin, Kip3 contains an N-terminal motor domain, an internal coiled-coil region, and a C-terminal 'tail' domain, the function of which is unknown. Here we report experiments showing the C-terminal tail domain has an inhibitory effect on Kip3's function. Kip3 alleles lacking the tail domain are hyperactive. Microtubules show increased shrinkage rate and more frequent catastrophe in the tail-less mutant cells than those in wild-type cells. Moreover, preliminary experiments suggest the recombinant tail-less kip3 is a more efficient microtubule depolymerase than the full-length kip3. In addition, the tail domain physically interacts with the motor domain in vitro. Taken together, this suggests an auto-inhibition mechanism of Kip3 regulation, which might be important for the cell-cycle remodeling of microtubule systems.

2614/B323

Active Zone Scaffolding Protein SYD-2 Regulates Kinesin-3 Activity.

S. Mandalapu[1], C. Thiede[1,2], S. Lakaemper[2,1], O. Wagner[4], B. Koehler[1], K. Shen[3], D. Klopfenstein[1]; [1]CMPB, Georg August University, Goettingen, Germany, [2]Drittes Physikalisches Institut, Georg August University, Goettingen, Germany, [3]Stanford University, Stanford, CA, [4]Institute Of Molecular and Cellular Biology, National Tsing Hua University, Hsinchu, Taiwan

Kinesins are molecular motors that carry out various intracellular transport processes along microtubule tracks. UNC-104 a C. elegans ortholog of human kinesin is one such molecular motor of the kinesin-3 family that is involved in transporting synaptic vesicles to synapses in C. elegans. UNC-104 belongs to the branch of monomeric motors. In vivo UNC-104 can transport vesicle cargo fast and processively. We hypothesize that UNC-104 interacting proteins might play a role in regulating transport activity. One molecular player that could fulfill this role is SYD-2. SYD-2 is important for the organization and architecture of a synapse by interaction with many scaffolding molecules at the active zone. Furthermore, SYD-2 binds UNC-104 thus making it a prime candidate for regulatory protein. We confirm their interaction by pull-down assays and measure the velocity and processivity of the motor in vitro in the presence and absence of SYD-2. Our preliminary results show that SYD-2 increases the velocity of UNC-104 in a multi-motor assay (microtubule surface gliding assay) as well as on a single molecule level (analysis by TIRF microscopy).

2615/B324

Stability of the C. Elegans Synaptic Vesicle Kinesin Motor, UNC-104 Co-relates with Specific Binding to Cargo.

J. Kumar[1], B. Choudhary[1], S. Naganathan[1], R. Metpally[2], S. Ramanathan[2], D. Klopfenstein[3], S. P. Koushika[1]; [1]Neurobiology, NCBS-TIFR, Bangalore, India, [2]Biochemistry, Biophysics and Bioinformatics, NCBS-TIFR, Bangalore, India, [3]Center for Molecular Physiology of the Brain, Georg August University, Göttingen, Germany

UNC-104 is a Kinesin-3 motor that transports synaptic vesicle proteins in C. elegans. This protein binds to PI(4,5)P2 on the cargo vesicle through its PH domain. The unc-104(e1265) allele carries a point mutation (D1497N) in its PH domain. This lesion is located close to a potential site for binding PI(4,5)P2 as observed in a homology model of the UNC-104 PH domain. As predicted, this lesion reduces specific binding to PI(4,5)P2 in vitro that results in the mutant UNC-104's inability to support synaptic vesicle transport and co-ordinated movement. The loss of specific cargo binding also results in reduced levels of the UNC-104 motor in vivo. Two intragenic suppressors isolated, tb107 and tb120, mapped within the PH domain. tb107 (R1501Q) mapped within the predicted lipid-binding pocket and tb120 (M1540I) mapped over 20Å away from the D1497N lesion in the homology model. Both suppressors partially restore specificity for PI(4,5)P2 binding in vitro co-related with improved vesicle transport in vivo and improved animal locomotion. tb107 and tb120 animals also have increased levels of UNC-104 protein compared to unc-104(e1265) animals. A conserved W1569 residue mediates the suppression of M1540I on D1497N. Surprisingly W1569A by itself is also able to suppress the effects of N1497. The W1569A D1497N variant of the UNC-104 PH domain also has partially restored specificity to PI(4,5)P2 in vitro. We observe that any change that abolishes specificity of the UNC-104 PH domain to PI(4,5)P2 in vitro also reduces its expression in vivo as assayed by full length GFP fusion proteins with the appropriate mutations in the PH domain. The only exception is the W1569A D1497N variant that does not express GFP in vivo but does provide partial rescue to locomotion and also partial restoration of in vitro binding specificity to PI(4,5)P2. These data suggest that inability to bind cargo leads to an unstable motor. Such a mechanism could be crucial to ensuring the stability of only motors that are specifically bound to their appropriate cargoes.

2616/B325

NCD Motor Binding and Transport in the Spindle.

M. Hallen, Z. Liang, S. Endow; Cell Biology, Duke University Medical Center, Durham, NC

The NCD kinesin-14 motor plays an essential role in meiotic spindle assembly in Drosophila oocytes and produces force in mitotic spindles that opposes other motors. Binding by NCD to spindle microtubules is thought to be mediated both by the highly charged tail and conserved motor domain, based on assays in vitro. However, little is know about how the motor binds to spindles in live cells, where binding could be modulated by interacting proteins. Here we analyze NCD motors deleted for the positively charged tail or conserved head, fused to a bright GFP, in live oocytes and embryos. We find that, unexpectedly, the tailless motor does not bind to oocyte meiotic spindles and binds poorly to mitotic spindles. By contrast, the headless motor binds to both meiotic and mitotic spindles, but the spindles are abnormal. Thus, the tail is essential for motor binding to the spindle, but both the head and tail are required for normal spindle assembly and function. We performed fluorescence recovery after photobleaching (FRAP) assays of the motors to estimate binding constants for NCD to the mitotic spindle. Strikingly, the recovery data for the headless and full-length motors did not fit well to previous models for fluorescence recovery. Analysis of fluorescence flow in early mitotic spindles showed movement of NCD from the pole towards the equator at the time the FRAP assays were performed. We derived a new model that differs principally from previous models in that it takes into account motion of the cytoskeleton and thus of bound NCD. It predicts two phases in recovery, a short early phase dominated by diffusion and a long later phase dominated by cytoskeletal motion that accounts for transport of NCD bound to the spindle in early mitosis. The new diffusion-binding-transport model yields binding rate constants that confirm the dominant role of the NCD tail in binding to the spindle, starkly contrasting with the weak binding by the motor inferred from fits of the tailless data to an existing model. The minus-end NCD motor thus binds tightly to spindles by its tail and is transported in early metaphase towards microtubule plus ends, the opposite direction as it moves, to produce force in the spindle later in mitosis.

2617/B326

Kinesin Involvement in FcγR - Mediated Phagocytosis.

K. Silver, R. E. Harrison; Cell and Systems Biology, University of Toronto, Toronto, ON, Canada

Fcγ Receptor (FcγR) mediated phagocytosis is a cellular event that is evolutionary conserved to digest IgG-opsonized pathogens. One of the microtubule motor proteins, dynein has been shown to be important in trafficking the nascent phagosome towards the central lysosomes for degradation by hydrolytic enzymes. We hypothesize a role for kinesin in forming the phagosome, by trafficking endomembrane vesicles and actin- regulating proteins to the localized phagocytic cup. Targeted membrane insertion has been suggested as a requirement for the ingestion of multiple/and or large particles. To investigate the role of kinesin in phagocytosis, cells were transfected with the dominant negative kinesin construct (Kif5B-DN). We observed a significant decrease in particle binding and internalization of sheep RBCs and large polystyrene beads (8 μm) in mouse RAW 264.7 macrophage cells transfected with Kif5B-DN. Immunostaining of lysosome-associated membrane protein (LAMP-1) revealed no defect in the maturation process of phagocytosis in cells transfected with Kif5B-DN. Overambitious macrophages engulfing a coated IgG glass surface were studied using epifluorescent microscopy and scanning electron microscopy in a 'frustrated phagocytosis' assay. Transfected Kif5B-DN cells engulfed half the area that control cells engulfed. To investigate the spreading defect further, focal adhesion proteins were studied because of their localization to the phagocytic cup. Focal adhesions in CHO2A FcγR-engineered phagocytic cells transfected with Kif5B-DN showed a central circular pattern and the cells were significantly smaller suggesting a role for kinesin in focal adhesion assembly at the cell border. The different focal adhesion pattern in transfected cells may be responsible for the spreading defect and possibly causing a decrease in actin polymerization. These results suggest that kinesin is a key player in phagocytosis by potentially delivering membrane or actin-regulating proteins to the phagocytic cup for the binding and internalization of opsonized particles.

2618/B327

Kinesin-1 Is a Signaling Protein That Recruits Active Kinase Complexes and Directly Participates in Substrate Phosphorylation.

Z. Muresan, V. Muresan; Pharmacology and Physiology, UMDNJ - New Jersey Medical School, Newark, NJ

To fulfill the functional requirements of the axon, the transport of the cargo to specific destinations along the axon is subjected to complex regulation, including the recruitment of specific kinesins to transport vesicles. Here, we investigated the signals that trigger the recruitment of kinesin-1 to vesicles containing the amyloid-β precursor protein (APP), a transmembrane protein with relevance to Alzheimer's disease that is transported into neurites. We previously showed that a fraction of the total APP within the cell body is phosphorylated at Thr^{668} by c-Jun NH_2-terminal kinase (JNK). This phosphorylated APP (pAPP) is transported to neurite terminals by recruiting kinesin-1 as part of a large complex that includes JNK and the JNK-interacting proteins-1 and 3 (JIP-3). Non-phosphorylated APP is also transported into neurites, independently of pAPP. Unlike pAPP, non-phospho-APP recruits kinesin-1 through Fe65, a modular protein that interacts with both APP and kinesin-1. Using the CNS cell line, CAD, we show that Fe65, JIP-3, and - surprisingly - kinesin-1 regulate phosphorylation of APP by JNK, and thus determine the fraction of APP that will be transported in phosphorylated or non-phosphorylated form. Overexpression of JIP-3 increases APP phosphorylation, while its downregulation by siRNA reduces phosphorylation. Also, overexpression of Fe65 sequesters APP and kinesin-1 at the endoplasmic reticulum (ER), and specifically increases the phosphorylation of the immature, ER-localized APP. Further, overexpression of the tetratricopeptide repeat domain of the kinesin light chains blocks transport of pAPP into neurites (through dominant negative effect), and also increases phosphorylation of APP. Overexpression of a mutant JIP-1 that competes with Fe65 for kinesin-1 binding prevents APP phosphorylation. These results support a model, where kinesin-1 serves as scaffold that recruits an active JNK/JIP-3 complex to APP-bound Fe65, leading to APP phosphorylation. Thus, kinesin-1 plays a direct role in intracellular signaling, which is not related to its motor function. Supported by NIH grant R01GM068596.

2619/B328

Transport of Proteolytically Processed Amyloid-B Precursor Protein Uses Kinesin and Myosin Motors and a Plethora of Scaffolding Proteins.

Z. Muresan, V. Muresan; Pharmacology & Physiology, UMDNJ - New Jersey Medical School, Newark, NJ

The amyloid-β precursor protein (APP), a transmembrane protein with relevance for the pathogenesis of Alzheimer's disease, undergoes proteolytic cleavage into defined fragments that are transported into axons by kinesin-1. The proteolytic cleavage of APP largely occurs prior to its sorting into axonal transport vesicles, and the cleaved fragments segregate into separate vesicle populations that reach different axonal destinations. The recruitment of kinesin-1 to these distinct vesicle populations most likely occurs through different mechanisms, and the transport is differentially regulated. Here, we tested this hypothesis with the mouse catecholaminergic cell line CAD. Using expression of dominant negative constructs, protein depletion by siRNA, and co-immunolocalization, we found that kinesin-1 is recruited to the vesicles containing different APP-derived polypeptides via distinct scaffolding proteins: JIP-1/JIP-3, Fe65, and DISC1. Specifically, kinesin-1 binds to the amyloid-β fragment-containing vesicles via DISC1, and to non-phosphorylated carboxy-terminal fragments (CTFs) via Fe65. The phosphorylated CTFs (pCTFs) recruit kinesin-1 via JIP-1, and are transported into neurites in large signaling complexes that include JIP-3 and activated JNK. Within neurites, the vesicles containing different APP fragments associate with different sets of microtubules and, occasionally, actin filaments: pCTFs were preferentially transported into neurites, and - unlike the amino-terminal and amyloid-β fragments - entered the lamellipodium and filopodia of growth cones, and concentrated in regions of growth cone turning and advancement. Within filopodia, pCTFs co-localized with actin filaments and myosin-X, an unconventional myosin known to undergo intrafilopodial motility. Accumulation of pCTFs at neurite terminals was diminished in cells treated with myosin-X-specific siRNA. Thus, the pCTFs are likely to switch from a microtubule-based transport (operating within the main process) to actin-based motility (operating in filopodia). We conclude that processing and transport of APP are fine-tuned, interdependent processes, which could determine the function of APP. Supported by NIH Grant R01GM068596.

2620/B329

Novel Role for Microtubule Motor Protein Eg5 in Ribosome Biogenesis and Protein Translation.

K. M. Bartoli[1], J. Jakovljevic[3], J. Woolford[3], W. S. Saunders[1,2]; [1]Biochemistry and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA, [2]Biological Sciences, University of Pittsburgh, Pittsburgh, PA, [3]Biological Sciences, Carnegie Mellon University, Pittsburgh, PA

Kinesin motor proteins are important for the transport of specific intracellular cargo along microtubules. In addition to their transport function, some kinesins have been implicated in mitosis and are critical for proper cell division. Eg5 is a plus-end directed microtubule motor that localizes to spindles during mitosis. Recently, we have observed that Eg5, and other kinesin motors, localized to the nucleolus of interphase cells. As the traditional function of the nucleolus is ribosome biogenesis, ribosome fractionation studies were conducted and demonstrated that Eg5 was found to associate with mature ribosomal subunits by sucrose gradient fractionation. Interestingly, inhibition of Eg5 function, either by a 24 hr knockdown or small molecule inhibitor, resulted in a stark decrease in protein translation. This observed decrease in protein translation was not due to a decrease in cell proliferation or to an increase in the mitotic index, both of which could result in decreased protein translation. Through polysome profiling studies, we have determined that the decrease in protein translation, after knockdown or inhibition of Eg5, is because Eg5 participates in translation elongation and the ATPase activity of Eg5 is essential for this function. Furthermore, inhibition of translation elongation was also demonstrated in the absence of microtubules. Moreover, through in vitro microtubule binding assays, we observed that a subset of ribosomes bind microtubules via Eg5 in mammalian cells and only ribosomes associated with the cytoplasmic fraction of the cell contain Eg5. Finally, we have observed Eg5 to localize to stress granules (SGs) containing stalled translation initiation complexes and mature 40S ribosomal subunits. Utilizing live cell imaging and immunofluorescence, we have determined in the absence of Eg5, a significant percentage (32%) of cells fail to form SGs, and those cells in which SGs are present form smaller, less motile SGs. Collectively, our data suggests a novel role for mitotic microtubule motor Eg5 in translational elongation.

2621/B330

The Homotetrameric Kinesin-5, KLP61F, Preferentially Crosslinks Microtubules into Antiparallel Orientations.

L. Tao[1], S. van den Wildenberg[2], L. Kapitein[2], C. Schmidt[3], E. Peterman[2], J. Scholey[1]; [1]Dept. of Molecular and Cellular Biology, University of California - Davis, Davis, CA, [2]Depts of Physics, Astronomy, and Laser Centre, VU University, Amsterdam, Netherlands, [3]Dept. of Physics, Georg August University, Göttingen, Germany

The mechanism of action of the mitotic spindle depends upon the polarity patterns of its constituent microtubules (MTs), and it is possible that mitotic motors play significant roles in establishing these polarity patterns. Here we have investigated this possibility by assaying the purified, Drosophila homotetrameric kinesin-5 motor, KLP61F, to determine if it displays a preference for crosslinking MTs of parallel or anti-parallel orientation. In motility assays, KLP61F was observed to be capable of crosslinking and sliding adjacent MTs, as predicted. In crosslinking assays with polarity-marked MTs, KLP61F displayed a three-fold higher preference for crosslinking MTs in the antiparallel, relative to the parallel orientation. This polarity preference was nucleotide-sensitive because it was observed in the presence of ADP or in ATP plus AMPPNP, but not in AMPPNP alone. Furthermore, it likely depended upon MT-binding by the C-terminal tail domain of KLP61F, since a purified KLP61F fragment lacking the motor domains also preferentially crosslinked antiparallel MTs. The results suggest that KLP61F prefers to crosslink MTs with anti-parallel overlap, which can then be moved apart if the motor is active. During the morphogenesis of the Drosophila embryo mitotic spindle, these combined activities could facilitate the gradual accumulation of KLP61F within antiparallel interpolar (ip) MTs at the equator, where the motor could then generate force to drive poleward flux and polepole separation. *L. Tao and S. van den Wildenberg are equal contributors.

2622/B331

MCAK Accumulates during the Cell Cycle and Is Degraded at the Metaphase to Anaphase Transition.

A. Ganguly, R. Bhattacharya, F. Cabral; Department of Integrative Biology and Pharmacology, The University of Texas Medical School at Houston, Houston, TX

Mitotic centromere associated kinesin (MCAK) belongs to a large family of microtubule-dependent motors, but it has the unusual ability to depolymerize microtubules and regulate microtubule dynamics. Although MCAK has been found to be present throughout the cell cycle, its main reported functions are to correct errors in chromosome alignment that may occur during prometaphase and to participate in anaphase sister chromatid segregation through a “Pac-man” like mechanism at chromosome kinetochores. Our studies of MCAK demonstrate that its lifetime closely matches the cell doubling time in 3 different mammalian cell lines, suggesting that it is degraded in cell cycle dependent manner. Using synchronized CHO cells, we further show that MCAK accumulates during most of the cell cycle, reaches a maximum at G2/M, and is rapidly degraded during mitosis. Immunofluorescence indicates that MCAK is present at spindle poles and kinetochores during prophase and prometaphase, but is largely absent once the chromosomes reach metaphase alignment. Addition of MG132 blocks the cells at metaphase and prevents the loss of MCAK from spindle poles and kinetochores, indicating that the proteasomal pathway degrades MCAK. The results are thus consistent with a role for MCAK during congression of chromosomes to the metaphase plate, but not with a role in anaphase chromosome movement. We have found that a phosphorylated form of MCAK that appears during mitosis seems to be preferentially degraded; but degradation does not appear to depend on Aurora B, a kinase reported to be involved in regulating the error correcting activity of the protein. We propose that a different kinase phosphorylates MCAK and targets the protein for degradation.

2623/B332

Kinesin-1-Driven Lipid Droplets: Consequences of Motor Copy Number for Intracellular Transport.

S. Gross[1], G. Schubeita[1], S. L. Tran[2,3], J. Xu[1], M. Vershinin[1], S. Cermelli[1], S. Cotton[3], M. Welte[2,3]; [1]Developmental and Cell Biology, UC Irvine, Irvine, CA, [2]Biology, University of Rochester, Rochester, NY, [3]Biology/Rosenstiel Center, Brandeis University, Waltham, MA

The microtubule motor Kinesin-1 plays central roles in intracellular transport, but how it is regulated is still poorly understood. *In vitro* work suggests that one means of controlling Kinesin-1-driven transport might be modulation of the number of Kinesins active per cargo since glass beads moved by multiple copies of Kinesins travel considerably further than those powered by single motors. Although it has been widely assumed that many cellular cargoes are indeed moved by multiple Kinesins and that cargoes with more motors move faster and for longer distances, concrete evidence for these notions is sparse, in large part because it is challenging to determine the number of motors active per cargo *In Vivo*. Here we analyze lipid-droplet motion in *Drosophila* embryos because we can employ optical tweezers to determine the force required to stall moving droplets; such stall forces provide a direct measure of motor activity. We first employ antibody inhibition, genetics, biochemistry, and particle tracking to demonstrate that Kinesin-1 mediates plus-end droplet motion. We confirm that decreasing embryonic Kinesin-1 levels leads to a concurrent decrease in droplet-bound Kinesin-1. We then measure how variation in Kinesin-1 expression affects the forces driving individual droplets and find that reducing the amount of Kinesin-1 per droplet results in a parallel decrease in stall forces. Our data provide an estimate for the number of Kinesins actively engaged per droplet and suggest that in the wild type most droplets simultaneously engage multiple Kinesins. They also allow us to address the relevance of motor copy number *In Vivo*. We find that in contrast to *in vitro* observations, increased motor number results neither in longer travel distances nor higher velocities. We conclude that cargo transport *In Vivo* is dominated not by motor number per se but by higher-order regulatory mechanisms.

2624/B333

Structural Model for Strain-Dependent Microtubule Activation of Mg-ADP Release from Kinesin.

R. Nitta, Y. Okada, N. Hirokawa; Cell Biology and Anatomy, University of Tokyo, Tokyo, Japan

Mg-ADP release is considered to be a critical process for the regulation and motility of the molecular motor kinesin. To gain insight into the structural basis of this process, we solved the series of the atomic structures of monomeric kinesin KIF1A during and after Mg release. On the basis of new structural data and mutagenesis data, we propose a model mechanism for microtubule activation of Mg-ADP release from KIF1A. In our model, a stereo-specific interaction between loop L7 of KIF1A and helix H4 of β-tubulin reconfigures the KIF1A active site by shifting the relative positions of switch I and switch II. This leads to the sequential release of a group of water molecules that sits over the Mg in the active site, followed by Mg, and finally the ADP. We further propose that this set of events is linked to a strain-dependent docking of the neck-linker to the motor core, which produces a two-step power stroke.

2625/B334

Characterization of the Drosophila Neuronal, Monomeric Kinesin, Khc-73.

T. M. Huckaba[1,2], A. Gennerich[1,2], S. Zimmerman[3,2], Y. Jan[3,2], R. D. Vale[1,2]; [1]Cellular and Molecular Pharmacology, University of California

San Francisco, San Francisco, CA, [2]Howard Hughes Medical Institute, Chevy Chase, MD, [3]Physiology, University of California San Francisco, San Francisco, CA

We have undertaken a biophysical, biochemical, cell biological, and whole-organism study to elucidate the mechanism of Khc-73 function, as well as its physiological role in the *Drosophila* nervous system. Using purified GFP-tagged Khc-73 constructs in single molecule motility assays, we observe a direct relationship between the length of the Khc-73 tail and the run length, as well as the frequency with which a motility event is observed, suggesting that small stretches of putative coiled-coil in the Khc-73 tail are able to facilitate dimerization. Khc-73 moves along axonemes *in vitro* at an average rate of 1.5 microns/second, and forced dimers of Khc-73 are able to move an average of 5 microns before dissociating. Gel filtration data using purified Khc-73 constructs suggests that even short constructs exist as dimers in solution. In optical trap studies, Khc-73 exhibits an average stall force of ~6 pN and takes on average 8 nm steps under a backward load. In *Drosophila* S2 cells, Khc-73 co-localizes with Rab5-containing vesicles in the cytosol and at cortical sites of Rab5 enrichment. Surprisingly, although Khc-73 co-localizes and co-immunoprecipitates with Rab5, it does not appear to be responsible for the motility of Rab5-contaning vesicles, as RNAi-mediated knockdown of Khc-73 and over-expression Khc-73 constructs lacking the motor domain have no effect on the rate or frequency Rab5-containing vesicle motility. However, the cortical enrichment of Rab5 is significantly reduced in similar conditions, suggesting that Khc-73 stabilizes a cortical Rab5 complex. Finally, to determine the role of Khc-73 in the *Drosophila* nervous system, we have constructed a series of Khc-73-GFP-expressing lines, as well as driven expression of Khc-73 RNAi. Expression of GFP-tagged Khc-73 in neurons exhibits axonal enrichment, although a smaller population can be observed in dendrites. Pan-neuronal expression of Khc-73 RNAi is not lethal, and no gross abnormalities in axonal outgrowth have been observed. Current work is aimed toward investigating any changes in Rab5 localization or deficits in known Rab5-dependent neuronal functions.

2626/B335

Comparison of the In Vitro Microtubule Depolymerising Activity of the Human and Malarial Kinesin-13 Motor Domain.

C. Hoey[1], E. Knuepfer[2], A. A. Holder[2], C. Moores[1]; [1]School of Crystallography, Birkbeck College, London, United Kingdom, [2]Division of Parasitology, National Institute for Medical Research, London, United Kingdom

Kinesins are a diverse family of proteins. They are ATP driven molecular motors that interact with microtubules (MT) and are characterised by their motor domain. This contains the MT and ATP binding sites necessary for their function. There are significant functional differences between kinesin families, but less is known about the cross-species variations within these families. The microtubule depolymerising properties of vertebrate full length kinesin-13s are well characterised in vivo and in vitro. However to understand the molecular basis of depolymerisation we have focussed on the biochemistry and mechanism of the motor domain alone. Vertebrate kinesin-13s regulate MT dynamics, particularly during mitosis and meiosis. Kinesin-13s are also active in a variety of parasites such as, Leishmania, Trypanosomes, and the malarial parasite; Plasmodium. The expression profile of the Plasmodium falciparum kinesin-13 (PfMCAK) shows it is predominant in the blood stages, and gametocytes of the malaria lifecycle. This suggests that, like its vertebrate counterparts, it plays a role in cell division. We have studied the differences between the structure and function of the motor domains from PfMCAK and human kinesin 13 (HsMCAK). Previous work using PfMCAK captured in the ATP state showed rings of MT protofilaments with kinesin motor heads bound, observed using an electron microscope. This provided the early model for how kinesin-13 destabilises MTs. However we observed the morphology of rings formed with HsMCAK is changed demonstrating a subtle difference in the mechanics between PfMCAK and HsMCAK despite a 75% homology between the motor domains. We also see that the depolymerising activity of PfMCAK is sensitive to MT architecture. It is unable to depolymerise MTs with low numbers of protofilaments while HsMCAK depolymerising activity remains unaffected by different protofilament architectures. ATPase studies of the two motor domains provide the biochemical perspective on kinesin-13 motor domain activity. Comparisons of kinesin-13 motor domains from phylogenetically distant organisms provide insight into the conserved and species-specific aspects of kinesin-13 activity.

2627/B336

Fission Yeast Kinesin-8: a Plus End-Directed Motor That Couples Microtubule Depolymerization to Cargo Movement.

P. M. Grissom[1], T. Fiedler[1,2], E. L. Grishchuk[1], D. Nicastro[1,3], R. West[1,4], J. McIntosh[1]; [1]MCD Biology, University of Colorado, Boulder, CO, [2]LCTech GmbH, Dorfen, Germany, [3]Dept. of Biology, Brandeis University, Waltham, MA, [4]Experimental Transplantation & Immunology Branch, National Cancer Institute, NIH, Bethesda, MD

Fission yeast expresses two Kinesin-8s previously identified and characterized as products of the klp5+ and klp6+ genes. Here we describe in vitro properties of these proteins expressed in either bacteria or Sf9 cells. The bacterially-expressed motor-plus-neck domain of Klp6p formed soluble dimers that bound and cross-linked microtubules in an ATP-sensitive manner as seen by co-sedimentation, light microscopy and cryo-electron microscopy. These dimers showed both microtubule-activated ATPase and plus-end directed motor activities. Full-length Klp5p and Klp6p were co-expressed in Sf9 cells to yield a soluble heterodimer that also cross-linked microtubules and had both ATPase and plus-end directed motor activities. Klp5FL/Klp6FL associated with the walls of MTs, forming bridges between adjacent MTs, as seen by both negative staining and cryo-electron microscopy. This heterodimer showed classic Michaelis-Menten kinetics with respect to both MTs and ATP. The Km, for MTs was 0.16 +/- 0.01 µM with a Kcat of 2.4 +/- 0.1 s-1 and the Km for ATP was 90.1 +/- 17.2 µM. Klp5FL/Klp6FL was also a motor in vitro, but its motility was sensitive to the concentration of added salt; MTs moved at an average speed of 0.56 +/- 0.08 µm/min in BRB80 buffer but at 2.35 +/- 0.20 µm/min in BRB80 plus 200 mM NaCl. Moreover, this kinesin-8 heterodimer could couple a cargo to the end of a depolymerizing microtubule, using the energy of depolymerization to pull a load in the minus-end direction. Klp5FL/Klp6FL coated latex microbeads followed the depolymerizing plus-end of MTs with a mean speed of 10.3 +/- 2.8 µm/min. Processivity was good (mean distance traveled was 7.2 +/- 5.9 µm). These results, together with the phenotypes of mutants of the kinesin-8s themselves and other interacting proteins, suggest that these polypeptides contribute to the coupling of spindle microtubules to chromosomes during mitosis.

2628/B337

DmKAP, the Accessory Subunit of Kinesin-2 Binds the Coiled-Coil Stalk of the Motor Subunits.

K. Ray; Tata Institute of Fundamental Research, Mumbai, India

Kinesin-2 is a plus end-directed microtubule-dependent motor complex of two different motor subunits and an accessory protein KAP3. The motor subunits were shown to bind each other through a coiled-coil domain in the middle while the KAP was proposed to attach through the tail domains (Wedaman *et al.,* JCB, 132:371, 1996; Yamazaki *et al.,* PNAS, 93:8443, 1996). Genetic studies also showed that KAP plays an important role in kinesin-2 functions. Thus, KAP was proposed to be the universal cargo adaptor for kinesin-2. Systematic co-purification studies with the recombinant fragments of Drosophila kinesin-2 subunits: KLP64D, KLP68D and DmKAP, suggested that the N-terminal domain of KAP is necessary and sufficient to bind the kinesin-2 motors. In addition, only the coiled-coil stalk domain of the motor subunits could bind the KAP. The interaction is stable at high salt concentration and sediments together in the sucrose density gradient. Furthermore, the tail domain of KLP64D alone is found to interact with choline acetyltransferase, a known axonal cargo of kinesin-2. Together with an in silico modeling study, this indicated KAP as a potential adaptor for certain coiled-coil domains necessary for assembling a greater complex involving kinesin-2. However, it is not necessary for interactions with all the kinesin-2 cargoes.

2629/B338

Collective Motility and Force-Generation of Exactly Two Coupled Kinesin-1 Motors.

M. Diehl[1,2], K. Jamison[2], A. Rogers[1]; [1]Chemistry, Rice University, Houston, TX, [2]Bioengineering, Rice University, Houston, TX

Systems of interacting motor proteins are known to be central to a variety of intracellular transport processes. However, efforts to understand the dynamic properties of motors when they operate collectively are largely inhibited by difficulties in determining the exact number of coupled motors that participate in motility both in vivo and in vitro. Objective: Our objective is to develop and characterize a biosynthetic system of coupled kinesin motors that emulate the structures of multi-component motor assemblies that are known to transport cargo in cells. Methods: Our assemblies contain exactly two coupled kinesin-1 motors separated by a distance of 50 nm. Assemblies are formed using a single duplex of DNA as a molecular scaffold and DNA-conjugated artificial polypeptides as capture probes that form selective and stable intermediate linkages between motor proteins and the scaffold through a self-assembly process. The successful construction of finite-sized multi-motor complexes then allows us to employ a series of 'single-molecule' assays to examine the collective motility and force generating properties of interacting motor molecules. Results: We find that current models of collective motor processivity are inadequate to describe the increased travel distances of interacting motors. Furthermore, we show that most motor assemblies detach from microtubules below the stall force of single kinesin motors. However, at high applied loads, two motors can produce more than twice the force of one. Conclusion: Our results point to a new model of collective motor transport where inter-motor strain and strain-dependent motor attachment/detachment kinetics dominate collective motor mechanics, resulting in inhibited motor function at low applied loads, but enhanced motor activity at high loads. We will discuss this model and new classes of experiments that are required to monitor the molecular orchestration in integrated systems of motor molecules and to resolve outstanding issues regarding the transport of cargo by multiple motors

2630/B339

Drag-Brake Mechanism of a Spindle Motor Kinesin-5.

K. Kaseda[1], K. Hirose[2], R. A. Cross[1]; [1]Molecular Motors, Marie Curie Research Institute, Oxted, United Kingdom, [2]Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology, Tsukuba, Japan

In most eukaryotic systems, kinesin-5 motors are absolutely required for mitosis and meiosis, where they drive engagement and subsequent sliding apart of the antiparallel half-spindles, whilst antagonising and/or collaborating with other motors. It was previously demonstrated that the kinesin-5 motor efficiently slows down other motors such as kinesin-1 and Ncd, suggesting it can generate resistive force as well as motive force (Crevel, I.M., Alonso, M.C., and Cross, R.A. 2004. Curr. Biol. 14, R411-412 and Tao, L., Mogilner, A., Civelekoglu-Scholey, G., Wollman, R., Evans, J., Stahlberg, H., and Scholey, J.M. 2006. Curr. Biol. 16, 2293-2302). Subsequent in vivo work has born this out: depletion of kinesin-5 causes faster spindle extension in Caenorhabditis elegans (Saunders, A.M., Powers, J., Strome, S., and Saxton, W.M. 2007. Curr. Biol. 17, R453-454) and causes up to 5 times longer and much more branched axons in neurons (Myers, K.A., and Baas, P.W. 2007. J. Cell Biol. 178, 1081-1091). The molecular mechanisms of these dual functions are poorly understood. Using mutagenesis, we show here that we can increase or decrease the drag-force component, with reciprocal effects on microtubule sliding velocity. In particular, we report a microtubule-binding-deficient mutant of Xenopus Eg5 with substantially reduced drag that slides microtubules 50% faster than wild type. Another mutant, in the kinesin-5 neck linker, shows increased drag together with decreased velocity. Our results suggest that whilst strongly-bound to the microtubule, Eg5 crossbridges are tuned to divide their time between force-generating states that drive microtubule sliding, and force-holding states that resist sudden or over-rapid microtubule sliding in cells.

2631/B340

How Kinesin Can Be So Processive.

E. Toprak[2], A. Yildiz[3], S. Rosenfeld[1], P. Selvin[2]; [1]Neurology, Columbia University, New York, NY, [2]Physics, University of Illinois, Urbana, IL, [3]Cellular and Molecular Pharmacology, University of California at San Francisco, Urbana, IL

Objective: To identify the key features of the mechanochemical cycle of kinesin I that allow it to walk over one hundred steps before detaching from its microtubule track. Methods: We had previously demonstrated that labeling kinesin I in the neck linkers with tetramethyl rhoadamine maleimide (TMR) generates a processive construct whose fluorescence emission is sensitive to the conformation of the neck linkers. We have now applied this technology to the single molecule level to measure the orientation of kinesin I on the microtubule at each point during a processive run and as a function of nucleotide concentration. We have combined the temporal resolution of this assay with the spatial resolution of fluorescence imaging with one nanometer accuracy (FIONA). Results: During each step of a processive run, the rhodamine fluorescence emission of TMR-labeled kinesin alternates between a high fluorescence ("unquenched") and low fluorescence ("quenched") state. The lifetimes of both states vary with ATP concentration, and modeling this variation and incorporating results from FIONA allows us to identify two features that significantly contribute to making kinesin processive. First, under physiological conditions,

kinesin is largely attached to the microtubule via both heads. This arrangement reduces the concentration of singly attached kinesin molecules, which would be susceptible to premature detachment, and insures that the predominant species is one that can experience intramolecular strain. Second, when kinesin is strongly bound to the microtubule via one head, its other head is capable of weakly interacting with the microtubule lattice—a feature that would further reduce the likelihood of premature detachment. Conclusions: Our model differs from two recently proposed mechanisms to explain kinesin processivity, in that it emphasizes the contributions of two key elements--strain-mediated gating of ATP binding and interaction of the unbound head with the microtubule surface—in making kinesin remarkably processive.

2632/B341

Direct Observation of Individual Head Motions in Kinesin Dimers.

N. Guydosh[1], S. Block[2,3]; [1]Biophysics, Stanford University, Stanford, CA, [2]Biology, Stanford University, Stanford, CA, [3]Applied Physics, Stanford University, Stanford, CA

Optical-trapping assays for dimeric kinesin typically involve attaching a bead to the common stalk of the molecule. The displacements of beads attached in this fashion report the average position of the entire molecule: motions executed by the individual heads during the stepping cycle remain unresolved. Here, we developed a novel single-molecule assay for tracking an individual head of Kinesin-1 under load, by attaching a bead to one of the two head domains via a short (70 bp) DNA tether. When hindering loads were applied, we observed distinct steps of ~16 nm, as anticipated for heads moving in a hand-over-hand walk. Under sustained assisting loads, we observed large jumps (>16 nm) in displacement at the start of step dwells as the applied load pulled the rear head forward, past its partner head and beyond the next microtubule binding site. Torque generated along the ~2-nm lever arm separating the points of DNA and neck linker attachment tends to rotate the DNA-linked head, explaining this 'overshoot' feature. ATP binding to the new rear head, which is known to trigger neck-linker docking, allows the front, DNA-linked head to rotate back to its normal orientation and bind the microtubule, yielding a net advance of 16 nm. We conclude that the rear head releases the microtubule prior to ATP binding to the front head. The newly-released head can then diffuse freely, tethered to its partner. ATP binding then causes docking of the neck linker to the microtubule-bound head and induces the partner head to bind the microtubule, becoming the new front head. We confirmed this sequence by applying a rapidly oscillating (hindering and assisting) load to kinesin during stepping and measuring the resultant difference in forward and rearward displacements of the bead. The magnitude of this signal varied between two discrete values, corresponding to those intervals when the DNA-linked head adopted bound and free states. The existence of a free state disfavors models where one head docks against its microtubule-bound partner between step transitions. We conclude that internal strain, generated whenever both heads simultaneously bind the microtubule, is responsible for gating the kinetic cycle to ensure kinesin processivity.

2633/B342

A Torque Component Present in Mitotic Kinesin Eg5 Revealed by Three-Dimensional Tracking.

J. Yajima, K. Mizutani, T. Nishizaka; Physics, Gakushuin Univ., Tokyo, Japan

Mitotic kinesin Eg5 (a member of the kinesin-5 sub-family of microtubule-based motors) has been shown to play a critical role during prometaphase and metaphase, in producing and maintaining a mitotic bipolar spindle architecture. Recently it has been reported that full length kinesin-5 tetramers can crosslink two antiparallel microtubules and move toward the plus-ends of both microtubules, indicating that several tetrameric kinesin-5 molecules drive relative sliding of microtubules. In optical trap experiments, kinesin-5 dimers have been demonstrated to be weakly mechanically processive, taking eight steps on average along a microtubule before detaching. A very recent contribution shows that single-headed kinesin-5 can drive microtubule sliding at almost the same speed as double-headed kinesin-5, indicating that processive walking is not necessary for kinesin-5 to be able to transport microtubules at close-to-wild-type rates. The extent to which kinesin-5 moves processively is thus currently controversial. In this study, we developed a novel assay that visualizes directly the corkscrew motion of a sliding microtubule in a motility assay, by three-dimensional nanometer-resolution optical tracking of a quantum dot attached to the microtubule. Our tracking method uses only one simple optical component, a prism, without modification of other aspects of a standard microscope. Using the method, we found that both surface-attached human Eg5 monomers and dimmers drove counterclockwise rotation of sliding microtubules around their axis with different pitches. These corkscrewing motions have not been seen previously for kinesin-5, and it demonstrates that single kinesin-5 heads generate torsional force as well as axial sliding force. We show that the rotational pitch of microtubule sliding can be used as a convenient indicator of the mechanical processivity, and we use our method to show that kinesin-5 dimers are somewhat processive, but less so than kinesin-1 dimers.

Actin Dynamics and Assembly II (2634 – 2655)

2634/B343

Phosphorylation of the Yeast Arp2/3 Complex Regulates Its Activity by a Distinct Mechanism from Acanthamoeba and Mammalian.

J. Haynes, L. LeClaire, D. Barber; Cell and Tissue Biology, University of California, San Francisco, San Francisco, CA

The established mechanism for stimulating actin nucleating activity of the Arp2/3 complex is binding of proteins belonging to the WASP/Scar family. However, we recently found that the Arp2/3 complex also must be phosphorylated to nucleate actin filaments and that phosphorylation of the Arp2 subunit on T237/238 or Y202 is necessary for lamella formation in cells (LeClaire *et al.*, 2008). Moreover, we showed that a requirement for phosphorylation is conserved from amoeba to mammals. Because Arp2 residues that are phosphorylated in higher eukaryotes are conserved in budding yeast, we asked whether phosphorylation of the yeast Arp2/3 complex is also necessary for its activity. We purified Arp2/3 complex by generating yeast cells expressing a tandem tagged version of Arp2 at endogenous levels and using a tandem affinity purification procedure. Using actin nucleation assays and the VCA-domain of Scar, we found that like *Acanthamoeba* and mammalian Arp2/3, untreated or mock-treated yeast Arp2/3 had robust actin nucleation activity, but complex dephosphorylated with a dual specificity phosphatase had almost no activity. We then tested whether Arp2 phosphorylation sites are conserved in yeast by making point

mutations in yeast Arp2 threonine and tyrosine residues that are required for Arp2/3 activity in other organisms. These *arp2* mutants grew normally, although Arp2 is essential for yeast cell viability. To determine which subunits are phosphorylated, we immunoblotted purified yeast Arp2/3 complex with anti-phosphothreonine and phosphotyrosine antibodies, which labeled Arp2 from *Acanthamoeba* but not from yeast. In contrast, the p34 subunit of yeast (Arc35) but not *Acanthamoeba* had a strong phosphothreonine signal, which was absent after phosphatase treatment. Our data indicate that similar to higher organisms, phosphorylation of the yeast Arp2/3 complex is necessary for activity; however, they suggest that the mechanism of regulated phosphorylation in yeast may be different.

2635/B344

Conformational Changes in the Arp2/3 Complex Induced by Phosphorylation.

A. Narayanan[1,2], L. LeClaire[3], D. Barber[3], M. P. Jacobson[2,4]; [1]Department of Pharmaceutical Chemistry, University of California San Francisco, San Francisco, CA, [2]Graduate Group in Biophysics, University of California San Francisco, San Francisco, CA, [3]Department of Cell and Tissue Biology, University of California San Francisco, San Francisco, CA, [4]California Institute for Quantitative Biosciences, San Francisco, CA

The Arp2/3 complex nucleates new actin filaments to construct branched filament networks that are key to a number of cellular processes including cell motility and endocytosis. Actin nucleation by the Arp2/3 complex requires interaction with a mother actin filament, ATP, and a nucleation-promoting factor. However, we recently reported (LeClaire et al., 2008 JCB) that Arp2/3 complex must also be phosphorylated to nucleate actin filaments and that phosphorylation of Arp2-Thr237/238 or -Tyr202 is necessary for lamella formation in cells. Although phosphorylation of Arp2 appears to cause or enable structural changes in the Arp2/3 complex that lead to activation, there are no details of the conformational changes that occur. To produce a structural model of the phosphorylated state of the Arp2/3 complex, we performed molecular dynamics simulations of the Arp2/3 complex in its unphosphorylated form or after in silico phosphorylation of Arp2-T237. We see large structural differences between the phosphorylated and unphosphorylated complexes, including changes in the relative orientation of the Arp2 and Arp3 subunits (15.7° rotation between average phosphorylated structure and crystal structure, compared with 7.8° for the average unphosphorylated structure) and in the distances between ARPC1 and ARPC3 (76Å in the average unphosphorylated structure measured between the Cα atoms of ARPC1 Val372 and ARPC3 Gly175, 90Å in the average phosphorylated structure, compared with 91Å in the crystal structure). We find that Arg106 from ARPC4 and Lys232 from Arp2 appear to form stable interactions with the phosphothreonine and may contribute to driving these conformational changes through the bending of the long α-helix in ARPC4. Because phosphorylation is insufficient for activation and the conformational changes observed are not on an obvious pathway toward the active state of the complex, phosphorylation may cause the Arp2/3 complex to adopt a distinct inactive conformation with a lower barrier to activation. Extending these simulations and simulations with phosphorylated Arp2-Tyr202 and site mutants can serve to generate testable hypotheses to aid further understanding of phosphoregulation of the Arp2/3 complex.

2636/B345

Nck-Interacting Kinase NIK Phosphorylates Arp2 to Increase Arp2/3-Mediated Actin Polymerization.

L. LeClaire[1], M. Baumgartner[1,2], D. Barber[1]; [1]Cell and Tissue Biology, University of California San Francisco, San Francisco, CA, [2]Clinical Research and Veterinary Public Health, University of Bern, Bern, Switzerland

The actin-related protein (Arp) 2/3 complex nucleates branched actin filament arrays in most crawling cells. We recently reported that the Arp2/3 complex must be phosphorylated on either tyrosine or threonine residues to be activated by N-WASP or Scar to nucleate actin filaments (LeClaire et al. 2008 JCB). We proposed that phosphorylation of the Arp2/3 complex at T237/238 or Y202 of Arp2 promotes a conformation change that allows activation by N-WASP or Scar. In studies to identify kinases that phosphorylate the Arp2/3 complex, we found that the Ste20-like Nck interacting kinase (NIK) directly phosphorylates the Arp2 subunit. Mammalian NIK and its orthologs Misshapen (Msn) in Drosophila and Mig-15 in C. elegans share evolutionarily-conserved roles in promoting cell migration determined primarily in animal models. However, little is known at the cell level about how these kinases enhance migration or cytoskeleton dynamics. NIK phosphorylates the Arp3, Arp2, and p35 subunits of the Arp2/3 complex in in vitro kinase assays. In pyrene actin polymerization assays, NIK phosphorylation of alkaline phosphatase-treated Arp2/3 complex restores nucleation activity of the complex. Consistent with stimulation of Arp2/3 actin nucleating activity, we found that GFP-NIK localizes to the leading-edge membrane protrusions in migrating fibroblasts. Additionally, RNAi suppression of NIK expression in fibroblasts and epithelial cells inhibits growth factor-stimulated lamellipodium formation and RNAi suppression of Msn in Drosophila S2 cells inhibits lamella formation. These data indicate that Arp2/3 complex subunits are substrates for NIK and they suggest that NIK increases phosphorylation of the Arp2/3 complex, actin polymerization, and lamellipodium formation to promote cell migration. Supported by Sandler Family Postdoctoral Research Fellowship to LLL and NIH GM58642 to DLB.

2637/B346

Coronin 1B Antagonizes Cortactin and Remodels Arp2/3-Containing Actin Branches in Lamellipodia.

L. Cai[1,3,2], A. Makhov[4,3], D. Schafer[5], J. Bear[1,3]; [1]Department of Cell and Developmental Biology, University of North Carolina at Chapel Hill, Chapel Hill, NC, [2]Department of Anatomy, University of California San Francisco, San Francisco, CA, [3]Lineberger Comprehensive Cancer Center, Chapel Hill, NC, [4]Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, NC, [5]Departments of Biology and Cell Biology, University of Virginia, Charlottesville, VA

The dendritic actin network generated by Arp2/3 complex in lamellipodia underlies formation of protrusions, directional sensing and migration. While the generation of this network is well studied, the mechanisms regulating network disassembly are poorly understood. We report that Coronin 1B disassembles Arp2/3-containing actin filament branches by inducing Arp2/3 dissociation. Using biochemical assays, we demonstrate that Coronin 1B potently inhibits the activation of Arp2/3 nucleation by Cortactin. In addition, Coronin 1B debranches nascent Arp2/3-containing actin branches, and competes with the branch stabilization activity of Cortactin. In addition, Coronin 1B promotes the dissociation of the Arp2/3 complex from the side of actin filaments, whereas Cortactin enhances this association. Consistent with the in vitro competition, depletion of both proteins partially rescues defects in lamellipodial dynamics observed upon depletion of either protein alone. Using TIRF and electron microscopy, we show that Coronin 1B specifically targets actin branches, and that Coronin 1B and the

Arp2/3 complex are mutually exclusive at branches suggesting that Coronin 1B has the ability to replace Arp2/3 complex at branches. The targeting of Coronin 1B to actin branches results in the alteration of the branch angle both in vitro and in vivo. Further, by dual-color live-cell imaging, we show that Coronin 1B targets pre-existing areas of Arp2/3 in lamellipodia, but persists longer at these sites than Arp2/3 complex during retrograde flow. Together, these observations indicate that Coronin 1B has the unique function of remodeling Arp2/3-containing actin branches. Coronin 1B replaces Arp2/3 complex at actin filament branches as the dendritic network matures and, together with Coronin 1B-mediated recruitment of the Cofilin-activating phosphatase, Slingshot-1L, drives the turnover of Arp2/3 branched actin networks. Although our studies have focused on lamellipodia, the mechanism of Coronin 1B function revealed by these studies likely applies to all Arp2/3-based branched actin networks such as those found on endosomes or phagocytic cups.

2638/B347

DNA-Scaffolded Protein Oligomers for Examining the Significance of Nano-Scale Geometry in Arp2/3-Mediated Actin Meshwork Assembly.

M. A. Tsuchida, M. J. Footer, J. Theriot; Biochemistry, Stanford University, Stanford, CA

The large-scale coordination of actin-based motility depends on the specific geometric organization of the factors involved in orchestrating the assembly and disassembly of actin filaments. Intracellular actin-based comet-tail motility of the Gram-positive pathogen Listeria monocytogenes has served as a model for branched actin meshwork assembly due to its amenability to in vitro assays and its simplicity: only a single bacterial protein, ActA, is required for motility of the bacteria in cell-free extract. ActA activates Arp2/3 complex, stimulating the branching and nucleation of actin filaments, and artificial microspheres coated with ActA can undergo motility in cell-free extracts. While the effects of the surface distribution, density, and mobility of ActA on the surface of motile objects has been characterized in detail, previous studies have been limited to examining or perturbing the overall distribution of ActA. We have recently shown that ActA on the bacterial surface is densely packed, and that artificially dimerized ActA possesses enhanced actin nucleation promoting activity (Footer et al., 2008, J. Biol. Chem., 283, 23852), suggesting that interplay or cooperation between closely tethered ActA molecules may be important in achieving stable meshwork polymerization. To further characterize the effects of the microscopic clustering of ActA, we have designed and synthesized monodisperse oligomers of ActA using a DNA scaffold. A modular design enables us to hybridize and purify clusters of several molecules of ActA, and the method has the potential to be extended to oligomers with defined, varied spacing and larger arrays of ActA with low polydispersity.

2639/B348

Real-Time Observation of Actin Branched Network Formation Prior to Actin-Based Motility.

V. Achard[1], C. Guérin[1], A. Michelot[1,3], L. Blanchoin[1], J. Martiel[2], R. Boujemaa-Paterski[1]; [1]iRTSV-CEA, Grenoble, France, [2]TIMC-IMAG, CNRS, Grenoble, France, [3]University California Berkeley, Berkeley, CA

To reach beyond simply defining the inventory of molecules that control actin dynamics and to understand how these proteins act synergistically to build branched actin network responsible for actin based motility, we combined evanescent-wave microscopy with a biomimetic system and followed the behavior of single actin filaments in the presence of a physiologically relevant mixture of accessory proteins. TIRF microscopy allowed us real-time visualization of actin branched network growing around WA-coated object. We showed that the ternary complex: Arp2/3 complex/actin filament/activator is a prerequisite for explosive actin polymerization around WA-coated object. The presence of capping proteins (CP) constrains actin assembly close to actin activators sites increasing the probability for the formation of this ternary complex and favors the formation of a branched network leading to symmetry breaking. According to our real-time experiments and modeling, the presence of an active polymerizing zone near WA-coated object pushes the surrounding capped filaments outwards. The resulting internal stress that builds up inside the network of capped filaments triggers its fracture and gives way to motility.

2640/B349

Observing Individual Actin Filaments and Branches in an In Vitro Reconstruction of Cell Motility: Activator Does Not Dissociate from Arp2/3 Branch Complexes Under Low-Load Conditions.

X. Hu, J. Kuhn; Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA

Although we know many of the reaction mechanisms involved in reorganizing the actin cytoskeleton for cell motility, we do not fully grasp how they are coordinated at an individual filament level. To date, in vitro reconstitutions of actin-based motility have focused primarily on the propulsion forces involved in bead or vesicle motility, and/or overall actin filament densities in the comet tail. We have developed a reconstituted motility assay that uses total internal reflection fluorescence (TIRF) microscopy to observe actin filament and Arp2/3 complex branch dynamics at the individual filament level. We use 200 nm thick glass nanofibers coated with GST-tagged VCA domain of the Arp2/3 activator N-WASP as model leading edges. In the presence of purified Arp2/3 complex, ADF/cofilin, capping protein, profilin, and fluorescent actin, activator-coated nanofibers greatly enhance Arp2/3 based nucleation at the fiber surface. By adjusting individual protein concentrations, we have observed individual filament dynamics rather than the overall actin gel. We found that branches are formed only at the nanofiber surface. No branches dissociated from the nanofiber during up to 2 hours of observation under these conditions, indicated that newly nucleated branches remain strongly attached to their activator. The persistent attachment between the Arp2/3 complex, filament branch, and N-WASP-VCA might be essential to maintain the high concentration of actin filaments to enhance further nucleation in the leading edge. In turn, locally increased actin filaments may keep N-WASP-VCA concentration high at the leading edge. Our results conflict with electron microscopic observations of highly branched dendritic network in actin comet tails far from the motile surface. As we did not observe symmetry breaking under low filament density and thus low pushing force conditions, physical force may be necessary to overcome the binding interaction either between the Arp2/3 branch complex and the activator, or the activator and the membrane.

2641/B350

SPIN90-IRSp53 Complex Is Important in Rac-Induced Membrane Ruffling and Positive Regulation of Rac.

C. Teodorof, J. Bae, J. Choi, W. Song; BioImaging Research Center and Cell Dynamics Research Center, Gwangju Institute of Science and Technology (GIST), Gwangju, South Korea

SPIN90 has been characterized as a protein that plays a role in actin cytoskeleton organization. Here, we identified IRSp53 as a binding partner of SPIN90 using BioGRID database (General Repository for Interaction Datasets). We confirmed the SPIN90-IRSp53 complex formation *In Vivo* and the direct association between SPIN90 proline-rich region and SH3 domain of IRSp53. The concomitant expression of SPIN90 and IRSp53 in COS-7 cells made SPIN90-IRSp53 complex to be translocated to the leading edges, strongly colocalizing on ruffles after PDGF treatment. In PDGF mediated membrane protrusions, IRSp53 required SPIN90 and RNA interference (RNAi) of SPIN90 reduced significantly lamellipodia-like protrusions caused by IRSp53 after PDGF treatment. The independent coexpression of SPIN90 and IRSp53 with activated Rac in COS-7 cells did not interfere with Rac-induced membrane ruffling and the inhibition of SPIN90-IRSp53 binding showed a dominant negative effect making more clear the role of SPIN90 in the ruffling formation. In addition, SPIN90 and IRSp53 synergistically activated Rac suggesting a positive interaction between SPIN90 and IRSp53 in the regulation of Rac.

2642/B351

The Wave Regulatory Complex Is Inhibited.

A. M. Ismail[1,2], S. B. Padrick[1,2], J. Umetani[1,2], M. K. Rosen[1,2]; [1]Biochemistry, UT Southwestern Medical Center at Dallas, Dallas, TX, [2]HHMI, UT Southwestern Medical Center at Dallas, Dallas, TX

The Wiskott Aldrich Syndrome protein family of proteins (WASP, N-WASP, WAVE 1-3), are regulators of actin dynamics. While WASP regulation by GTPases and kinases is well characterized both biochemically and structurally, little is known about WAVE regulation. WAVE exists as part of a five protein complex termed the WAVE Regulatory Complex (WRC). It consists of WAVE, Sra, Nap, Abi and HSPC300. Biochemical studies of WRC have been hampered by the difficulty of expressing WRC components in bacterial, insect or yeast expression systems. Baculoviruses yielding high expression of each component of WRC were obtained using a modified pFastBac vector. Co-infection into Sf9 cells allowed efficient expression and purification of WRC and two sub-complexes, Sra-Nap and Abi2-WAVE1-HSPC300. We show here that WRC is inactive toward Arp2/3 complex in pyrene based actin assembly assays. However, Abi2-WAVE1-HSPC300 heterotrimer is active and Sra-Nap heterodimer inhibits it suggesting that WRC is autoinhibited. A modified WRC complex, where WAVE1 has a PreScission protease site between its VCA domain (the active domain) and its N-terminus and is lacking the polyproline region, is also inactive toward Arp2/3 complex. However, upon digestion with PreScission protease, this modified complex becomes active and the two products (VCA and the rest of the complex) can be separated readily by gel filtration. This suggests that the affinity of Sra-Nap heterodimer to VCA is inherently weak and the heterodimer requires the linkage provided by the Abi2-WAVE1-HSPC300 heterotrimer to VCA to efficiently inhibit it from activating the Arp2/3 complex. The same results are obtained using all Drosophila components. Finally, NMR line broadening experiments determined that the heterodimer binds VCA predominantly in the V and C regions. This would prevent VCA from binding both actin (binds to V) and Arp2/3 complex (binds to C and A), providing a competition model for the inhibition mechanism.

2644/B353

The Arp2/3 Activator WASH Controls the Fission of Endosomes through a Large Multiprotein Complex.

E. Derivery[1,2], B. Bugyi[1], J. Gautier[1,2], M. Carlier[1], A. Gautreau[1,2]; [1]Lab Enzymologie et Biochimie Structurales, CNRS, Gif-sur-Yvette Cedex, France, [2]CNRS UMR144, Institut Curie, Paris, France

Actin assembly drives fundamental cellular processes including cell motility and membrane remodeling. The Arp2/3 complex when activated by Nucleation Promoting Factors (NPF) generates branched meshworks of filaments. Recently a new family of NPFs called WASH has been identified, but its cellular role is unknown. Here we show that GFP-WASH and endogenous WASH are localized in dynamic perinuclear structures containing the Arp2/3 complex and F-actin in 3T3 cells. These structures are associated with one side of transferrin-containing endosomes, that were characterized as sorting/recycling endosomes. This suggests that WASH-mediated actin polymerization might propel endosomes, as previously reported for the N-WASP NPF. However, depletion of WASH by RNAi does not prevent the movement of endosomes, but rather induces a striking change in their shape, with numerous narrow tubules spreading out from these organelles. These tubules, which elongate on microtubules, are likely the result of defective membrane fission of nascent vesicules, since WASH can be specifically immunoprecipitated with dynamin and that dynamin inhibition produces similar tubules. WASH is constitutively active suggesting that like WAVE NPFs, it might be regulated by a multiprotein complex. Accordingly, several proteins were copurified with WASH from a stable HEK293 line expressing a tagged version of WASH. Two of these were identified as the CapZ heterodimer, a capping complex that inhibits barbed end elongation of actin filaments. Capping inhibitory activity is known to be essential for the generation of Arp2/3 dependent actin meshworks. However, it is to our knowledge unprecedented that the capping protein is part of the same macromolecular assembly as the NPF. Our results thus identifies a novel molecular machinery connecting actin polymerization to membrane fission at the level of endosomes.

2645/B354

EFC/F-BAR Proteins and the N-WASP-WIP Complex Bind to Membranes to Induce Membrane-Curvature Dependent Actin Polymerization.

S. Suetsugu[1,2], K. Takano[1]; [1]IMCB, Univ Tokyo, Tokyo, Japan, [2]PRESTO, JST, Saitama, Japan

Shapes of membrane dynamically change in various cellular phenomenon such as endocytosis and membrane protrusions. Membrane-binding domains including BAR and F-BAR/EFC domains are combined with protein-protein-interaction domain such as Src-homology 3 (SH3) domain. Recently, biochemical and structural analyses demonstrated that membrane-binding domains including BAR and EFC/F-BAR domain sense membrane curvature. However, it is unclear whether full-lengths of these membrane-binding proteins determine the place of actin polymerization dependent on curvature of membrane itself. Here we show that FBP17 or Toca-1 induces actin polymerization through the N-WASP-WIP complex and the Arp2/3 complex dependently on curvature of liposomes. The curvature-dependent actin polymerization was mediated by the membrane-interactions of FBP17 or Toca-1 protein and N-WASP of the N-WASP-WIP complex. The spatial organization of these proteins to the membrane was precisely regulated in curvature-dependent actin polymerization.

2646/B355

Out of Equilibrium In Vitro Actin Networks.

S. Koehler, K. M. Schmoller, C. Semmrich, O. Lieleg, A. R. Bausch; E27: Lehrstuhl fuer Zellbiophysik, TU Muenchen, Muenchen, Germany

Out of equilibrium actin structures are major components of the eukaryotic cytoskeleton. However, so far mainly equilibrated *in vitro* actin networks are utilized to study the effect of actin cross-linking or bundling proteins on structural and mechanical network characteristics. ATP-dependent processes (e. g. induced by molecular motors) will alter these properties *In Vivo*. Using confocal microscopy, we show that myosin II indeed can rearrange and even destroy actin bundles formed by fascin. These activities are sensitive to the ATP-concentration as well as to the number of motors acting simultaneously on an individual actin bundle. Furthermore, we show that ATP hydrolysis is not compulsory to obtain a non-equilibrium state - already the cross-linking protein filamin alone arranges actin filaments into kinetically trapped structures. Similarly to α-actinin/actin networks, the actual microstructure is determined by an aggregation controlled growth process, which in the case of filamin additionally results in the exhibition of internal stresses. Treadmilling processes are not sufficient to allow for a reorganization of these highly static and stable networks. Non-equilibrated cytoskeletal structures may provide cells with a valuable tool to facilitate the formation and reorganization of a broad variety of local patterns without significantly changing its constituents.

2647/B356

A New Model Explaining Temperature Dependence of Actin-Based Cell Motility.

G. Allen[1], A. Mogilner[2], J. Theriot[1]; [1]Department of Biochemistry, Stanford University, Stanford, CA, [2]Department of Neurobiology, Physiology, and Behavoir, University of California Davis, Davis, CA

Cell motility is a complex process requiring the combined activities of several macromolecular components. Even in cichlid keratocytes, perhaps the simplest of the well studied crawling eukaryotic cell types, the mechanisms that determine cell speed are unknown. In this study, keratocyte speed was measured over a temperature range of 5 to 40 degrees Celsius and was found to follow the Arrhenius equation, which models the temperature dependence of a chemical reaction. This suggests either that actin-based cell motility has a single rate-limiting step or that there are multiple rate limiting steps of similar activation energy. Detailed measurements on single cells (n= 30) showed coefficients of determination (R2) of 0.75 +/- 0.14 to the Arrhenius equation. Interestingly it was found that the apparent "activation energy" for cell speed, which for a single chemical reaction should be a fixed constant from experiment to experiment, varies with the speed of the cell at 25 C. This phenomenon has been described previously in the literature as rate compensation between enthalpy and entropy. However, a more concise explanation could also arise by modeling the rate-limiting step as a simple mixture of multiple processes with similar activation energies. Putative rate-limiting steps can be targeted by pharmacological manipulation. Stabilizing actin filaments with jasplakinolide had no effect on activation energy (n=3); while inhibition of myosin-II mediated contractility with blebbistatin (n=10) increased the activation energy of motility (p=0.02). This suggests that myosin II activity is one of several rate-limiting processes of similar activation energies that determine cell speed. Arrenhius relationships have been found previously for other highly complex cellular phenomenon. This model provides an explanation for their occurrence and predicts the appearance of rate compensation due to a complex admixture of multiple reactions with similar activation energy.

2648/B357

Biochemistry on a Leash: the Roles of Tether Length and Geometry in Signal Integration Proteins.

D. A. Van Valen[1], R. Phillips[1], M. Haataja[2]; [1]Applied Physics, California Institute of Technology, Pasadena, CA, [2]Department of Mechanical and Aerospace Engineering, Princeton Institute for the Science and Technology of Materials (PRISM), Princeton University, Princeton, NJ

We use statistical mechanics and simple ideas from polymer physics to develop a quantitative model of proteins with flexibly tethered ligands and receptors that control the activity of these proteins. We predict how the properties of tethers influence the function of these proteins and demonstrate how their tether length dependence can be exploited to construct proteins whose integration of multiple signals can be tuned. One case study to which we apply these ideas is that of the Wiskott Aldrich syndrome proteins (WASPs) as activators of actin polymerization. More generally, tethered ligands competing with those free in solution are a common phenomena in biology, making this an important specific example of a widespread biological idea.

2649/B358

An Agent-Based Model of Cytoskeletal Dynamics at the Cell Cortex.

R. Gile[1,2], J. Polka[1,3], D. Strickland[1,4], E. Munro[1,2], J. Alberts[1,2]; [1]2008 Physiology Course, Marine Biological Laboratory, Woods Hole, MA, [2]Center for Cell Dynamics, University of Washington, Friday Harbor, WA, [3]Biochemistry and Cell Biology, UCSF, San Francisco, CA, [4]Biochemistry and Molecular Biology, University of Chicago, Chicago, IL

Cells use actomyosin-based contractile networks to perform an astonishing variety of different tasks during early embryonic development—to polarize, to divide, to move and to participate in tissue level morphogenesis. Rather than building independent machines to do each of these jobs, cells redeploy the same network, tuning its physical properties to match the task at hand. Although the regulatory mechanisms by which cells tune actomyosin contractility are diverse and complex, it is possible that these dramatic changes are effected by modifying a few common control points: the dynamics of actin filaments; the extent to which the filaments are cross-linked; and the activity of myosin motors. To address this hypothesis, we have developed an agent-based computational model of an actomyosin network and used it to explore how contractile dynamics observed during polarization and cytokinesis in the *C. elegans* zygote might emerge from local interactions among actin filaments, cross-linkers and myosin motors. We represent these elements as discrete agents that experience thermal forces, bind to one another, and exchange forces according to explicit rules. Filaments nucleate, treadmill and are subject to capping and severing. Cross-linkers reversibly bind and transmit forces between nearby filaments. Myosin II mini-filaments bind and translocate actin filaments. We find that tuning the properties of filaments, cross-linkers and motors yields a rich variety of organizations and mesoscale contractile behaviors, comparable to those observed at the cortex of *C. elegans* zygotes during early development. In particular, we find that the model can reproduce a characteristic behavior that underlies polarity establishment in the zygote, in which myosin-rich contractile foci form, move and then disassemble.

2650/B359

Biased Gliding of Magnetized Actin Cytoskeleton in Applied Magnetic Field.

Y. Chen[1,2], R. Conroy[1], J. Sumner[1], J. Moreland[2], A. Koretsky[1]; [1]NINDS, National Institute of Health, Bethesda, MD, [2]National Institute of Standard and Technology, Boulder, CO

Actin cytoskeleton determines the shape of the cell, cell motility, the intracellular transportation of organelles/molecules and many more cell physiology. Being able to regulate/manipulate the dynamics of actin filaments can be key to control cell behaviors. In this study, we first magnetized the actin filament by conjugating the filament with 10 nm (in diameter) magnetic particles through biotin-streptavidin bond, in order to apply magnetic field to manipulate its directionality. We demonstrated that magnetic nanoparticles-loaded actin filaments can glide on the myosin-coated Permalloy chip or glass. When magnetic force was applied to the actin gliding surface, preliminary analysis showed biased gliding direction, when close to the magnetic pole, according to the magnetic field orientation. By introducing fluorescent magnetic particles bound to actin-binding proteins into the cells, we are now trying to establish an in vitro magnetic actin cytoskeletal network that can be controlled by applying magnetic forces. Because of the fluorescent labeling of the magnetic particles, the manipulation process can be monitored in dual imaging modality, optical microscopy and MRI, depending on the temporal/spatial scale of the specific cell behavior to be studied.

2651/B360

AKAP-Independent Localization of Protein Kinase a to Dynamic Actin Structures.

R. L. Rivard, A. K. Howe; Pharmacology, University of Vermont, Burlington, VT

Background: Regulation of the cyclic AMP-dependent protein kinase (PKA) in subcellular space is required for cell migration and cytoskeletal dynamics. Currently, spatial regulation of PKA is thought to require the association of PKA regulatory (R) subunits with A-kinase anchoring proteins (AKAPs). Here, we show that the regulatory RIIα subunit of PKA associates with dynamic actin microspikes in an AKAP-independent manner. RIIα co-localizes with microspikes in neuronal growth cones and leading edge lamellae and live-cell imaging demonstrates that RIIα−associated microspikes are highly dynamic. The association is selective, as only a subset of highly dynamic microspikes show co-localization with RIIα. Also, the coupling of RIIα to actin is very close, as demonstrated by the immediate and coincident inhibition of microfilament and RIIα dynamics with low-dose cytochalasin D. Importantly, co-localization of RIIα and actin in these structures is resistant to displacement by a cell-permeable disrupter of PKA-AKAP interactions. Biochemical fractionation confirms that a substantial pool of PKA RIIα is associated with the detergent-insoluble cytoskeleton and that this association is resistant to extraction by both an peptide inhibitor of AKAP interactions and by high-dose cytochalasin D. Finally, mutation of the AKAP-binding domain of RIIα fails to disrupt its association with dynamic actin microspikes. These data show that PKA closely associates with dynamic actin structures and provide the first demonstration of AKAP-independent subcellular localization of PKA RIIα. This association may have important consequences for microfilament dynamics and cell migration and may prime the investigation of novel mechanisms for specifying PKA activity within cells.

2652/B361

Changes in Myoid Cell Actin Network by Contraction.

L. Lopez[1], M. V. Bertoldi[1], J. C. Cavicchia[1], A. Morales[1], E. Callegari[2]; [1]IHEM, UNCuyo, Mendoza, Argentina, [2]Division of Basic Biomedical Sciences, The Sanford School of Medicine of the University of South Dakota, Vermillion, SD

In the mammalian testis, peritubular myoid cells (PMCs) surround seminiferous tubules (STs). These cells are contractile and express the cytoskeletal markers of true smooth muscle, such as alpha-isoactin and F-actin, and participate in the contraction of seminiferous tubules during the transport of spermatozoa and testicular fluid to the rete testis. The goal of this work was to analyze the changes in the PMC actin network induced by contraction. ST contraction was assayed by incubating isolated STs with or without 50 nM endothelin-1 or 100 nM oxytocin in MEM for 20 sec at 22 °C, followed by immediate fixation with 4 % paraformaldehyde in PBS. STs were incubated with anti-alfa actin-FITC conjugated antibody. The samples was mounted with mowiol and analyzed in a confocal inverted microscope. For actin filaments analysis 10 sequential fluorescent images of the same field were acquired in successive 1 µm z axis displacements as we scanned through the PMC layer in the wall of ST with a Plan Apo N60 X objetive. The z series of fluorescence section were analyzed with a Image J program to obtain a Z-stack image of maximal intensity proyection. The Z-stack image of PMCs from unstimulated STs (control) showed the characteristic orthogonal meshwork of actin filaments. While in STs stimulated with endothelin-1, the actin filament network evinced undulating actin filaments, losing the orthogonal organization characteristic of control PMCs. This pattern of actin filament organization was observed in the whole ST wall, but differed from previous descriptions where endothelin-1 induced an actin filament network reduced in size, and organized in bundles in a centrally located regions of the cell. To the best of our knowledge, this is the first time that a Z-stack image of the actin network from PMCs is described, and shows different details of actin organization compared to conventional light microscopy. Meanwhile, PMCs from oxytocin stimulated STs showed the typical orthogonal network of actin filament found in PMCs of control STs.

2653/B362

Identification of Genes That Regulate Actin Cable Stability in Budding Yeast.

T. Lipkin, J. Y. Lui, T. Huckaba, L. Pon; Pathology and Cell Biology, Columbia University, New York, NY

In *Saccharomyces cerevisiae* actin cables are bundles of F-actin, which lie along the mother-bud axis. They are essential for the establishment and maintenance of cell polarity in yeast, in part, because they serve as tracks for intracellular movement of organelles, secretory vesicles, mRNAs and other cargos. Previous efforts have revealed the protein constituents of actin cables, including two tropomyosins (Tpm1p and Tpm2p), fimbrin (Sac6p) and an actin bundling protein (Abp140p), as well as a role for the formin proteins (Bni1p and Bnr1p) in stimulating actin polymerization during actin cable assembly and elongation. Previous studies have also revealed that in yeast, actin cables are selectively destabilized by low levels of the actin destabilizing drug Latruculin-A (Lat-A). Here, we describe the results of a screen designed to identify gene deletions that reduce the sensitivity of yeast to the growth inhibiting effects of low doses of Lat-A. Out of 4,848 deletion strains comprising the yeast deletion library, 24 secondary screen hits (0.5% of the collection) exhibit reduced sensitivity to

Lat-A: 21% of these hits represent previously uncharacterized open reading frames, 17% represent cytoskeletal or polarity factors, and 17% represent vacuolar sorting proteins. We find that 21 of the hits (87.5%) exhibit an increased number of actin cables, as compared to the wild-type strain. Overexpression of Tpm1p results in an increase in actin cable number. In contrast, deletion or expression of constitutively active formins has no effect on actin cable number. These findings reveal a role for previously uncharacterized genes in the regulation of actin cable stability, as well as provide new roles for previously characterized, conserved genes in this process.

2654/B363

Polycation Induced Formation of Actin Filament Bundle.

A. Muhlrad[1], E. E. Grintsevich[2], M. Phan[2], E. Reisler[2]; [1]Institute of Dental Sciences, Hebrew University, Jerusalem, Israel, [2]Department of Chemistry and Biochemistry and the Molecular Biology Institute, University of California Los Angeles, Los Angeles, CA

Actin bundles have an important role in the formation of the cytoskeleton and in cell motility. Polycations, including polycationic proteins and polyamines, are known to polymerize negatively charged actin monomers and bundle actin filaments by screening negative charges and neutralizing repulsive interactions between the filaments. The stability of polycation induced actin bundles decreases with increasing ionic strength. We studied the formation and stability of actin bundles in the presence of three polycations, the 19-mer polylysine, a natural polyamine spermine and polycationic protein lysozyme, in light scattering and low speed sedimentation experiments. Roughly equivalent concentrations of polylysine and lysozyme to actin protomers were needed to bundle the filaments, while higher concentrations of spermine, which has a lower valency, were needed to bundle actin. Bundling of filaments by polylysine (as followed in a stopped flow apparatus) consists of a fast and a slow phase, with 0.84 and 13.2 s half life times, respectively. Dissolution (unbundling) of the polylysine-induced bundles by 150 mM NaCl is much faster than their formation, with a half life time of 5.03 ms. The ionic strength sensitivity of the bundles depends on the polycation used for their formation and decreases in the order spermine, lysozyme, and polylysine. In bundles formed by these three polycations antiparallel dimers (AD) were detected by cross-linking Cys-374 residues on two actin protomers with the MTS-1 disulfide reagent. These cross-linked dimers, which inhibit the dissolution of bundles by NaCl, originate most likely from adjacent actin molecules on oppositely oriented filaments in the bundles.

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Actin Bundling: Initiation Mechanisms and Kinetics.

P. Kraikivski, B. Slepchenko, I. Novak; University of Connecticut Health Center, Farmington, CT

Bundling of rapidly polymerizing actin filaments underlies dynamics of filopodial protrusions that play an important role in cell migration and cell-cell interaction. Recently, formation of actin bundles has been reconstituted *in vitro* and two scenarios of bundle initiation, involving binding of two filament tips and, alternatively, linking of the tip of one filament to the side of the other, - have been discussed. A first theoretical analysis is presented indicating that the two mechanisms can be distinguished experimentally. While both of them result counter-intuitively in comparable numbers of bundles, these numbers scale differently with the average bundle length. We propose an experiment for determining which of the two mechanisms is involved in the *in vitro* bundle formation.

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CaM Kinase II Initiates Spindle Depolymerisation in Xenopus Independently of APC/C Activation.

O. J. Gruss[1], S. Reber[1], S. Over[1], I. Kronja[2]; [1]ZMBH-DKFZ Alliance, Universitaet Heidelberg, Heidelberg, Germany, [2]Cell Biology and Biophysics Programme, EMBL, Heidelberg, Germany

Changes in spindle microtubule dynamics in anaphase are the basis for chromosome segregation. In Xenopus egg extracts, the trigger for anaphase onset is an increase of free Calcium and a subsequent rise in Calcium-Calmodulin-dependent kinase II(CaMKII) activity. CaMKII promotes the activation of the Anaphase promoting complex (APC/C), which destines securin for degradation and consequently allows chromosome separation. Here, we investigated how the depolymerisation of microtubules, concomitant with chromosome movement, is induced in anaphase of Xenopus extracts. Using RanGTP- induced microtubule assemblies as well as quantitative analysis of complete spindles, we show that CaMKII triggers anaphase microtubule depolymerisation, which can be explained by a twofold increase in microtubule catastrophe rates. We show that Calcium and CaMKII can even promote microtubule destabilization at high cdk1 activity or inhibition of the APC/C. Our data demonstrate a novel, CaMKII-dependent mechanism, which is responsible for the destabilization of spindle microtubules, and works in parallel to APC/C activation at anaphase onset.

2657/B368

The Unique N-Terminus of the UbcH10 E2 Enzyme Controls the Threshold for APC Activation and Enhances Checkpoint Regulation of the APC.

M. Summers[1], B. Pan[2], K. Mukhyala[3], P. Jackson[1,4]; [1]Cellular Regulation, Genentech, Inc, South San Francisco, CA, [2]Protein Engineering, Genentech, Inc, South San Francisco, CA, [3]Bioinformatics, Genentech, Inc, South San Francisco, CA, [4]Pathology, Stanford University School of Medicine, Stanford, CA

The Anaphase Promoting Complex (APC) E3 ligase requires an E2 to ubiquitinate substrates. In vitro, the APC functions with E2s of the E2-C and Ubc4 families. However, in organisms bearing a clear family member, only the use of the E2-C family, notably UbcH10, is genetically supported. We have used a combination of in vitro and in vivo techniques to examine the preferential use of UbcH10 by the APC. The in vivo specificity between the APC and UbcH10 is mediated by the E2 core domain. Importantly, an additional E2-E3 interaction mediated by the N-terminal extension of UbcH10 regulates APC activity. Mutation of the conserved N-terminus allows ubiquitination of APC substrates lacking their destruction-boxes, increases ubiquitination and the number of substrate lysines targeted, and increases resistance to the APC inhibitors Emi1 and BubR1 in vitro and the spindle checkpoint in vivo. Fusion of the N-terminus restricts the activity of other E2s, but does

not direct specific interactions with the APC. Thus, by requiring proper engagement for substrate ubiquitination, enforced via the N-terminal extension, the specific use of UbcH10 is a critical determinant of APC activity and checkpoint control.

2658/B369

Mitotic Dynamics of p31comet: Turnover at Unattached Kinetochores and Dynamic Associations with the APC/C.

M. S. Manak[1,2], H. Kirkeby[1,2], Y. Guan[1,2], A. Brear[1], J. V. Shah[1,2,3]; [1]Renal Division, Brigham and Women's Hospital, Boston, MA, [2]Systems Biology, Harvard Medical School, Boston, MA, [3]Division of Health Sciences and Technology, Harvard-MIT, Boston, MA

The mitotic checkpoint functions to ensure that chromosomes are not segregated until they all form bipolar attachments to the mitotic spindle. The target of the checkpoint is the anaphase-promoting complex/cyclosome (APC/C) that is inhibited from degrading securin and cyclin B while kinetochores remain unattached. Mad2, BubR1, and Bub3 have all been shown to turnover at unattached kinetochores implicating them in checkpoint signaling. Moreover, these proteins form a complex known as the mitotic checkpoint complex (MCC) with Cdc20 that acts to inhibit the APC/C in mitotic cells. In order to study the dynamics of p31comet a negative regulator of the MCC at the kinetochore, PtK2 cells were stably integrated with a p31comet-YFP fusion gene. Results from the current study using fluorescent recovery after photobleaching measurements of p31comet turnover at unattached kinetochores indicate that p31comet is a transient component of the kinetochore reporting the absence of microtubule attachment to the cytoplasm similar to Mad2. Based on the rapid onset of anaphase after the last kinetochore attachment and p31comet turnover at the kinetochore, we propose that p31comet becomes inhibited at the unattached kinetochore rendering it unable to dissociate the MCC-APC/C inhibitory complex. After complete attachment p31comet is reactivated, permitting the activation of the APC/C. To investigate the nature of the inhibition, HeLa cells constitutively expressing TAP-tagged p31comet were generated and mitotic lysate purifications were subjected to mass spectroscopy to identify p31comet-interacting proteins and sites of possible p31comet phosphorylation. Predicted phosphorylation sites were mutated to investigate their effect on mitotic progression. The recognition of the dual inhibitory role of kinetochores reconciles the biological observations of tight inhibition of the APC/C in the presence of a single unattached kinetochore with the rapid onset of anaphase after complete attachment. These measurements have been incorporated into a computational model that can account for these switch-like dynamics.

2659/B370

Identification and Functional Analysis of the Microtubule-Interacting Transcriptome in Embryonic Stem Cells.

J. A. Sharp, M. D. Blower; Molecular Biology, Massachusetts General Hospital, Boston, MA

RNA localization is of critical importance in many cellular processes for regulating the spatial control of gene expression. Work from our laboratory has demonstrated that a specific subset of mRNAs, including mRNAs that function in developmental patterning and cell fate specification, are significantly enriched on spindle microtubules (MT-RNAs) in Xenopus egg extracts, suggesting that the spindle partitions these mRNAs to ensure proper delivery to daughter cells during cell division of the early embryo. The mechanisms by which embryonic stem (ES) cells maintain cellular identity during cell division is a fundamental question in stem cell biology. The transcriptional program of ES cells is known to be distinct from somatic cell types, but the mechanisms that maintain ES cell-specific mRNA pools during cell division remain largely uncharacterized. To determine if MT-RNAs can act to maintain cellular identity in ES cells, we have developed an in vitro spindle assembly system to identify MT-RNAs. Mitotic ES cell extracts trigger the formation of radial microtubule asters organized around a central core structure enriched with γ-tubulin. In addition, we can detect the specific coprecipitation of factors important for microtubule nucleation, dynamics, and pole focusing, including γ-tubulin, Ch-TOG, Kinesin-13, and NuMA. These data indicate that ES cell mitotic asters formed in vitro are enriched with factors required for normal structure and function of the mitotic spindle. We find that ES cell MT-RNAs represent about 1-3% of the total RNA. In Xenopus, polyribosomes are associated with microtubules, and actively translate a subset of MT-RNAs. Both rRNA and tRNA coprecipitate with ES cell asters, as well as the rRNA-binding protein L7a, suggesting that some mRNAs present in the MT-RNA population are engaged with the translational machinery. We are currently using deep sequencing methods to access the full spectrum of ES cell MT-RNAs. Identification of MT-RNAs in ES cells and differentiated somatic cells will reveal whether different classes of mRNAs characterize the MT-RNA population of different cell types, with the potential to discover novel regulators of stem cell pluripotency and viability.

2660/B371

The Role of Microtubule-Induced Activation of Aurora B in Spindle Assembly.

B. Tseng, H. Funabiki; Chromosome and Cell Biology, the Rockefeller University, New York, NY

Chromatin-induced spindle assembly in *Xenopus* egg extracts requires the chromosomal passenger complex (CPC), which is composed of the kinase Aurora B, INCENP, Dasra (also known as Borealin), and Survivin. The Aurora B pathway is activated by chromatin, and enrichment of the complex on chromosomes is mediated by Dasra and Survivin. Activated Aurora B promotes local microtubule stabilization. The Aurora B pathway, however, can also be stimulated by microtubule stabilization. Unlike chromatin-induced activation of Aurora B, this microtubule-induced pathway does not require Dasra and Survivin. The mitotic role of this microtubule-induction is currently unclear. Here we present evidence indicating that both the microtubule-induced activation and chromatin-induced activation are required for spindle assembly. Interestingly, using a mutant of INCENP that lacks the coiled-coil domain, we show that chromatin-induced activation of Aurora B was insufficient for spindle assembly. While activation of Aurora B by chromatin was not affected, this INCENP mutant was unable to activate Aurora B induced by microtubules and was unable to rescue spindle assembly around chromatin. Furthermore, while an artificial activation of Aurora B using anti-INCENP antibodies can bypass the necessity for the chromatin-induced activation pathway in spindle assembly, the artificial activation of Aurora B could not rescue the spindle formation defect of this INCENP mutant. These results suggest that while chromatin is required to prime the Aurora B pathway and stabilize microtubules around chromosomes, further positive feedback between microtubules and the CPC plays a critical role in robust spindle formation.

2661/B372

AuroraB/Ipl1 Regulates Microtubule Plus-Ends via Cluster Phosphorylation of the EB1 Homolog Bim1p in Budding Yeast.

T. Zimniak, K. Stengl, K. Mechtler, S. Westermann; Research Institute of Molecular Pathology, Vienna, Austria

EB1-like proteins are conserved regulators of microtubule dynamics in all eukaryotes. They target growing microtubule-plus ends in vivo and deliver associated factors to regulate the interactions of microtubules with cellular structures such as kinetochores or cortical attachment sites. Despite their widespread importance, molecular mechanisms regulating the activity of these proteins have largely remained elusive. Here we show that the budding yeast EB1 protein Bim1p is regulated by AuroraB/Ipl1 phosphorylation of its linker domain. Bim1p directly interacts with the Ipl1/Sli15 complex via its C-terminal cargo-binding domain and becomes phosphorylated on a cluster of 6 sites in the flexible linker region connecting the conserved CH (Calponin-homology) and EB1-like domains. Recombinant Bim1p phosphorylated with Ipl1/Sli15 in vitro or a mutant mimicking constitutive phosphorylation display a reduced affinity for taxol-stabilized microtubules. Hydrodynamic analysis suggests that phosphorylation changes the conformation of the dimeric Bim1p molecule and alters its affinity for microtubules. We have reconstituted the interaction between Bim1p and dynamic microtubules using total internal reflection fluorescence microscopy (TIRF) in vitro. Bim1p autonomously tracks growing microtubule plus ends in vitro and this activity requires the dimeric molecule. The flexible linker region significantly contributes to microtubule binding and cluster phosphorylation creates an autoinhibitory domain that competes with tubulin for binding of the CH domain. These results suggest a model for the regulation of an EB1 protein by a mitotic kinase and have implications for the regulation of kinetochore-microtubule attachments in vivo.

2662/B373

Structures of Kinetochore Microtubule plus Ends from Yeasts to Mammals, Visualized by Electron Tomography.

J. McIntosh[1], E. L. Grishchuk[1,2], E. O'Toole[1], A. K. Efremov[1,3], K. V. Zhudenkov[4], D. Mastronarde[1], F. I. Ataullakhanov[5]; [1]Department of Molcular/Cell/Developmental Biology, University of Colorado, Boulder, CO, [2]Institute of General Pathology and Pathophysiology, Moscow, Russian Federation, [3]National Research Center for Hematology, Moscow, Russian Federation, [4]Physics, Moscow State University, Moscow, Russian Federation, [5]Theoretical Problems of Physicochemical Pharmacology, RAS, Moscow, Russian Federation

Recent work with electron tomography has identified fibrils that connect flaring protofilaments (PFs) at the plus ends of kinetochore microtubules (KMTs) to the inner kinetochore (McIntosh et al., Cell, in press). These slender structures modify the curvature of the PFs with which they associate, suggesting that they bear tension and convey force from shortening KMTs to kinetochores, thereby contributing to poleward chromosome motion. Here we describe results from analogous studies of additional strains of mammalian cells, blastomeres of the nematode, C. elegans, the green alga, Chamydomonas and both fission and budding yeasts. In spindles from all these organisms only mammalian chromosomes show an outer "plate," but all species show flared PFs at the plus ends of KMTs in both metaphase and anaphase spindles, and all show indications of "kinetochore fibrils" that connect flared PFs with chromatin. Quantitative analysis of PF curvature shows systematic differences between the PFs of yeasts and other organisms, suggesting that factors other than fibrils may contribute to the mechanics of PF bending and its coupling to chromosome movement in fungal cells. Work is ongoing to align KMT plus ends from multiple chromosomes in the same physiological state in a quest for repeatable structures that may correlate with these altered PF configurations.

2663/B374

Laser Ablation of Mitotic Microtubules and the Resulting Kinetcs of Mitosis in Male Rat Kangaroo (PTK2) Cells.

D. L. Flores[1], N. Baker[1], L. Shi[1], M. Berns[1,2,3]; [1]Bioengineering, University of California San Diego, La Jolla, CA, [2]Biomedical Engineering, University of California Irvine, Irvine, CA, [3]Beckman Laser Institute, University of California Irvine, Irvine, CA

During cell mitosis, the spindle fiber microtubules play a key role in the kinetics of pole and chromosome movement. Using the automated RoboLase II laser microscope(Botvinick and Berns, 2005, Micros. Res. Tech. 68:65-74),microtubules of single ECFP-tubulin transfected male rat kangaroo (PTK2) cells were ablated during the metaphase stage of mitosis. The irradiation of 0.2447 nJ/micropulse, corresponding to an irradiance of 1.4496*10^7 watts/cm2, produced well-defined micro-destruction of mitotic microtubules. The ensuing response of the poles and mitotic spindle were followed using fluorescent microscopy and the RoboLase II system. The results include several different patterns of kinetics in both the pole whose microtubules were ablated, as well as the pole which received no biophotonic alteration. Both the pattern of ablation and the type of microtubules targeted (astral vs. kinetochore) effected the resulting kinetics. These findings may help elucidate the sensory and structural mechanisms of spindle collapse and force generation during mitosis.

2664/B375

The Drosophila Dgt6 Protein Interacts with Msps/ XMAP215 to Promote Kinetochore-Driven MT Formation.

E. Bucciarelli[1, 2], C. Pellacani[1], V. Naim[1], A. Palena[1], M. Gatti[2], M. Somma[1]; [1]Ist Biol e Pat Mol CNR, Rome, Italy, [2]Dip. Genetica e Biol. Mol., Università La Sapienza, Rome, Italy

We analyzed the mitotic role of Dgt6, a member the *Drosophila* augmin complex required for spindle formation. Dgt6 exhibits a highly dynamic localization pattern; it is enriched at the center of prophase asters, at the kinetochore fibers (k-fibers) and at the central spindle. RNAi-mediated knockdown of Dgt6 resulted in spindles with a low MT density lacking robust k-fibers. These spindles were mostly unable to mediate sister chromatid separation but, nonetheless, they managed to elongate and assume ana-telophase configurations. To define the role of Dgt6 in kMT formation, we analyzed MT regrowth in prometaphases/metaphases following cold-induced MT depolymerization. In control cells returned to room temperature for 15-30 seconds, we observed short, randomly oriented k-fibers enriched in both Dgt6 and Msps. In Dgt6-depleted cells, k-fiber formation after return to room temperature was severely impaired, suggesting that the aberrant spindles observed in these cells are generated by a primary defect in kinetochore-driven k-fiber assembly. We also observed that Msps and D-TACC fail to accumulate at the centrosomes in *Dgt6* RNAi cells. Consistent with these results, we found that Dgt6 co-immunoprecipitates with Msps, D-TACC and the kinetochore component Ndc80/Hec1. Collectively, our results suggest a model on the role of Dgt6 in spindle assembly. We propose that Dgt6 binds dynamic MTs and recruits Msps/XMAP215, which in turn promotes rapid MT polymerization by catalyzing the addition of tubulin dimers to the growing plus ends.

2665/B376

Response of Mitotic Spindles to Mechanical Force.

S. Dumont[1,2], T. Mitchison[1]; [1]Department of Systems Biology, Harvard Medical School, Boston, MA, [2]Harvard Society of Fellows, Harvard University, Cambridge, MA

Cell division is an inherently mechanical process, from chromosome congression, error correction and segregation to furrow ingression. Although the molecules involved in cell division are becoming better characterized, surprisingly little is known about the underlying mechanical principles and interactions. Here we develop an assay to mechanically flatten mitotic spindles in live mammalian cells and use fluorescence microscopy to monitor the response of the microtubule cytoskeleton and kinetochores. We show that, upon flattening, the spindle deforms asymmetrically, widening quickly and passively but elongating more slowly in an active, tubulin polymerization-dependent manner. Analysis of spindle and cortex deformation, microtubule fiber shape and drug perturbations suggests that the components responding to this mechanical perturbation and driving spindle elongation are internal to the spindle, rather than a deformed cortex pulling the spindle.

2666/B377

Tension Is Not Required for Satisfying the Kinetochore Attachment Checkpoint.

Z. Yang[1], D. Brito[2], C. Rieder[1,2]; [1]Wadworth Center, N.Y.S. Dept. of Health, Albany, NY, [2]Biomedical Sciences, State University of New York, Albany, NY

During mitosis the kinetochore attachment checkpoint (KAC) is not satisfied until all sister kinetochores are stably attached to spindle microtubules (MTs). Tension between kinetochores is also necessary for inactivating the KAC, but it is controversial whether tension is sensed directly or if it is an upstream event required for the saturation of kinetochores with MTs. We recently noted that the duration of mitosis in human RPE-1 cells averages 12 hrs in 0.5 µM Taxol but only 3 hrs in 5 µM Taxol, and that 99% of the cells survive mitosis in 5 µM compared to 73% in 0.5 µM Taxol. The same is also true for BJ fibroblasts. Since mitosis is prolonged for >12 hrs when RPE-1 cells are treated with 5 µM taxol after inhibiting spindle MT assembly with 1 µM vinblastine, 5µM Taxol accelerates mitotic exit by its effects on MTs and not a secondary target. Cyclin B/GFP degradation kinetics revealed that RPE-1 cells treated with 5 µM Taxol do not undergo mitotic slippage but instead they satisfy the KAC. Subsequent fixed cell studies revealed that the inter-kinetochore distance in RPE-1 cells treated with 5 or 0.5 µM Taxol was the same, and similar to that of untreated prophase cells. Thus although a complete lack of tension between sister kinetochores in 0.5 µM Taxol leads to a durable (12 hr) mitotic delay a similar lack of tension in 5 µM Taxol does not. Together these data reveal that tension is not required for satisfying the KAC. We are now using 3D EM studies to test the hypothesis that 5 µM, but not 0.5 µM, Taxol promotes checkpoint satisfaction by inducing all sister kinetochores to become saturated with MTs in the complete absence of MT dynamics.

2667/B378

External Tension Controls Cell Division Axis via Retraction Fibres.

J. Fink[1], A. Betard[2], F. Luc[2], A. Azioune[1], N. Carpi[1], M. Bornens[1], M. Thery[3], D. Cuvelier[2], M. Piel[1]; [1]UMR 144 CNRS, Institut Curie, Paris, France, [2]UMR 168 CNRS, Institut Curie, Paris, France, [3]iRTSV - Laboratoire Biopuces, Biopuces, CEA, Grenoble, France

Proper positioning of the cell division axis is crucial for cell fate, development and tissue organization. We use adhesive fibronectin micropatterns to control cell adhesion and thereby cell shape, polarization and mitotic spindle positioning[1] in vitro. We recently assumed that retraction fibres which connect the mitotic cell body to the adhesive substrate are responsible for activating cortical force generators that pull on the astral microtubules. As a consequence the spindle poles will rotate towards the zones of increased retraction fibres density[2]. To directly challenge this theoretical model we designed two approaches to micromanipulate the retraction fibres: biphoton ablation and stretching on flexible substrates - both combined with micropatterns. When we removed retraction fibres partially by biphoton ablation, the spindle poles moved towards the remaining fibres, showing rotations of up to 90 degrees. Secondly we applied a 30 percent stretch on mitotic cells spread on a flexible substrate. We found that the shape of the mitotic body was hardly affected whereas the retraction fibres were notably straightened and elongated in the direction of the stretch. Perpendicular to this stretch the fibres became shorter and slightly bended. Remarkably the spindle poles rotated towards the zones of elevated tension. We believe that the transmission of the tension increase/release to the mitotic cell body is responsible for the observed spindle rotation. Cells were able to divide normally after biphoton ablation or after stretching. In this work we provide experimental proof that the retraction fibres are involved in positioning the mitotic spindle in human cells (RPE1 and HeLa). We conclude that retraction fibres serve in mechanotransduction throughout mitosis by transmitting the external tension towards the mitotic cell body and thereby providing important cues for the spindle orientation. The role of Src kinases during this process is under investigation, since they are known to be important for mechanosensing in interphase. 1.Thery, M., et al. Nat Cell Biol (2005). 2.Thery, M., et al. Nature (2007).

2668/B379

The Role of PPH-6 in Modulation of Pulling Forces for Spindle Positioning in One- Cell Stage C. Elegans Embryos.

K. Afshar, P. Gönczy; Life Science, Swiss Institute for Experimental Cancer Research (ISREC) and Swiss Federal Institute of Technology Lausanne (EPFL), Lausanne, Switzerland

Spindle positioning is key for generating cellular diversity during development. In the *C. elegans* one-cell stage embryo, the mitotic spindle is positioned asymmetrically toward the posterior during metaphase and anaphase, resulting in unequal division. Spindle displacement follows from an imbalance of pulling forces on the spindle poles, with more active force generators acting on the posterior pole. Microtubule dynamics, dynein, and a Galpha protein cassette comprising the Galpha subunits GOA-1, GPA-16, the Goloco protein GPR-1/2 and the NuMA homologue LIN-5, are required for production of pulling forces. Nevertheless, mechanisms underlying modulation of the pulling forces in space and time are unknown. We have been investigating the role of the Serine-Threonine phosphatase PPH-6 as a modulator of pulling forces. Depletion of PPH-6 causes defects in Pseudocleavage, chromosomes congression and spindle oscillations during anaphase. Spindle severing experiments indicates that PPH-6 acts as a positive regulator of pulling forces during anaphase but not metaphase. To investigate the mechanism of action of the PPH-6 as a positive regulator of pulling forces, we examined the dynamics of microtubules and

the acto-myosin cytoskeleton using spinning disc confocal microscopy of YFP-Tubulin and GFP-NMY-2. These experiments allowed us to uncover that PPH-6 is needed for the clustering of NMY-2 that normally takes place during anaphase (Werner et al, 2008). Furthermore, we found by mass spectrometry that PPH-6 is in a complex with Aurora A homologue AIR-1. Interestingly, upon depletion of PPH-6, AIR-1 becomes localized to the cortex throughout the cell cycle, whereas its localization to centrosomes and asteral microtubules is normal, suggesting that PPH-6 somehow negatively regulates cortical localization of AIR-1. Overall, our results suggest a role for PPH-6 in the regulation of acto-myosin network, which may itself be regulated by AIR-1 and be related to the generation of pulling forces during anaphase.

2669/B380

The Nucleo-Cytoplasmic Ratio Determines the Efficiency of the Spindle Assembly Checkpoint in Sea Urchin Embryos.

J. Kreher, D. Miranda, B. Rios, c. Shuster; NMSU Biology Department, New Mexico State University, Las Cruces, NM

The cleavage period of early development is characterized by a rapid increase of cell number with a concomitant decrease in cell size. The requirement for rapid cell cycles comes at the cost of cell cycle checkpoints, which guard the integrity and transmission of the genome. The spindle assembly checkpoint, which monitors chromosome attachments to the mitotic spindle, is only weakly effective in early embryos, but the true efficacy of the checkpoint has not been titrated in whole embryonic cells. To study checkpoint responses during early embryogenesis, we used eggs and early embryos of the sea urchins Strongylocentrous purpuratus and Lytechinus pictus. Examination of published mircoarray data as well as our own RT-PCR analyses revealed that mRNAs for the major checkpoint genes are present in the early embryo. To test the hypothesis that the nucleo-cytoplasmic ratio (NCR) determines the efficacy of the spindle checkpoint, we measured the length of mitotic arrest of control diploid or polyspermic eggs treated with microtubule poisons. Alternatively, we also measured the length of mitotic arrest in checkpoint-activated eggs where the NCR was increased by physical micromanipulation. Measurements of eggs with varying degrees of polyspermy revealed that increased numbers of sperm (ie chromosomes and kinetochores) linearly lengthened the time in mitosis. Similarly, measurements of eggs with reduced cytoplasm also exhibited an increase in the length of mitosis relative to control eggs. Lastly, measurements of blastomeres resulting from asymmetric cell divisions revealed that the length of mitotic arrest in micromeres was drastically longer than that observed for the paired macromere. Together, these results suggest that embryonic cells do have a functional mitotic checkpoint, whose efficacy is determined by the nucleo-cytoplasmic ratio.

2670/B381

Beyond Focal Adhesions: Novel Targets and Functions of Integrin-Linked Kinase (ILK) In the Centrosome and Mitotic Spindle.

S. Dedhar; BC Cancer Research Centre/UBC, Vancouver, BC, Canada

Integrin-Linked Kinase forms a multi-protein complex within focal adhesions and regulates integrin-mediated actin cytoskeletal organization and cell signaling. ILK is essential for embryonic development and it also plays critical roles in organ and tissue homeostasis. Overexpression and activation are however frequent hallmarks of many types of cancers. Recent proteomic based approaches to isolate ILK binding partners have now identified ILK as also being a component of interphase and mitotic centrosomes, where it associates with tubulin and tubulin-associated proteins. Inhibition of ILK activity or depletion by gene silencing causes mitotic spindle defects. ILK is essential for regulating Aurora A/TACC3/chTOG interactions required for mitotic spindle assembly. We have now applied phospho-proteomic approaches to identify potential targets of ILK within the mitotic spindle. Inhibition of ILK activity or ILK gene silencing results in the inhibition of phosphorylation of several mitotic spindle proteins. Of particular interest are Zyxin, a focal adhesion protein that also localizes to the mitotic spindle; TPX2 targeting protein for Xkip2, which is a Aurora A kinase activator and is essential for spindle pole organization; and NUSAP, nucleolar and spindle pole-associated protein, which stablizes microtubules and is also essential for mitotic spindle organization. ILK regulates phosphorylation of Zyxin on Serine 344, and ILK and zyxin co-localize in focal adhesions and mitotic spindles. Another mitotic spindle kinase, Nek-2 ,directly phosphorylates ILK, and hyperactive Nek-2 induces profound mislocalization and peri-nuclear accumulation of ILK. Overexpression of ILK and Aurora A kinase, induce resistance to Paclitaxel whereas small molecule Aurora A kinase inhibitors synergize with ILK inhibitors to induce mitotic spindle defects, mitotic arrest and cell proliferation. ILK therefore plays critical roles in coordinating major cytoskeletal interactions via the actin and microtubule cytoskeletal networks, and our investigations have uncovered unexpected novel roles of ILK in mediating microtubule organization during mitosis by regulating the functions of essential mitotic spindle proteins.

2671/B382

A Requirement of Nudel and Dynein for Spindle Matrix Assembly during Spindle Morphogenesis.

L. Ma[2,1], M. Tsai[1,4], S. Wang[1], B. Lu[3], R. Chen[1], J. R. Yates[3], X. Zhu[2], Y. Zheng[1,2]; [1]Embryology, Carnegie, Baltimore, MD, [2]Biochemistry and Cell Biology, Shanghai Institute, Shanghai, China, [3]Cell Biology, The Scripps Research Institute, La Jolla, CA, [4]Eppley Institute for Research in Cancer and Allied Diseases, Omaha, NE

Guanosine triphosphatase Ran (RanGTP) can stimulate assembly of the type V intermediate filament protein, lamin B, into a membranous spindle matrix, which is required for proper spindle assembly. We report that the isolated spindle matrix contains known spindle assembly factors such as dynein and NudEL. We show that both dynein and NudEL regulate spindle morphogenesis by promoting assembly of the spindle matrix. NudEL directly interacts with lamin B to facilitate the accumulation and assembly of lamin B on microtubules in a dynein-dependent manner. Further analyses using two spindle assembly assays in Xenopus egg extracts showed whereas disrupting either NudEL or dynein inhibited assembly of lamin B into the spindle matrix, depletion of lamin B still allowed formation of matrices containing dynein and NudEL. This suggests that lamin B functions downstream of NudEL and dynein to regulate proper microtubule organization during spindle assembly. Consistent with this idea, we found that whereas depletion of lamin B resulted in disorganized spindle poles, disrupting the functions of NudEL or dynein resulted in a complete lack of spindle pole focusing. Our studies demonstrate that NudEL regulates microtubule organization during spindle assembly independent of its function at kinetochores. One function of NudEL in microtubule organization is to recruit lamin B onto microtubule and facilitate the assembly of lamin B containing spindle matrix in a dynein dependent manner. Key words: Spindle matrix, lamin B, NudEL, dynein, cell fate determinants, membrane partitioning.

2672/B383

Centrosome Asymmetry in *Drosophila.*

P. Conduit, J. Raff; Gurdon Institute, Cambridge, United Kingdom

Centrosomes are composed of centrioles surrounded by pericentriolar material (PCM), which harbours sites for microtubule nucleation. Centrosomes duplicate and separate once per cell cycle in order to organise and align the mitotic spindle. Cells that divide asymmetrically rely on correct spindle alignment to properly segregate cell fate determinants. In certain asymmetrically dividing *Drosophila* cells centrosomes behave asymmetrically and this is important for centrosome positioning and spindle alignment. It is still unclear, however, what causes centrosome asymmetry in *Drosophila* because electron microscopy has not identified any structural differences between older (mother) and younger (daughter) centrioles. The aim of this study was to investigate the mechanisms involved in creating centrosome asymmetry in *Drosophila*. We find that all centrosomal components tested so far are asymmetrically distributed between separating mother and daughter centrioles in the syncytial embryo where centrosome asymmetry would not be expected. By using the fluorescence level of a GFP- centriole localisation domain fusion protein to estimate relative centriole age, we show that centrosome asymmetry exists because older centrioles have an intrinsic ability to retain larger amounts of protein. One of the proteins tested, D-Plp, is also asymmetrically distributed between mother and daughter centrioles in asymmetrically dividing neuroblasts. Our results reveal that a 'molecular clock' mechanism exists in the *Drosophila* syncytial embryo where centriole age is associated with protein retention capacity. This mechanism appears to be preserved in asymmetrically dividing *Drosophila* cells and may help to ensure that the mother centrosome is 'captured' and held in the correct position, whilst the daughter is free to move around the cell in order to set up a correctly aligned bipolar spindle.

2673/B384

The Centrosome Plays an Essential Role during Bipolar Spindle Assembly in Cells with an Intact Interphase Microtubule Network.

J. Hornick[1], C. Mader[2], E. Tribble[3], E. Halpin[1], E. Hinchcliffe[1]; [1]Biological Sciences, Univ. Notre Dame, Notre Dame, IN, [2]Molecular Biophysics and Biochemistry, Yale University, New Haven, CT, [3]Cell and Developmental Biology, Univ of North Carolina, Chapel Hill, NC

Bipolar mitotic spindles can form in experimentally derived acentrosomal cells - spindle assembly being driven by chromosomal associated factors. However, in these experimental studies, spindles assemble from randomly oriented microtubules (Mts) interacting with chromatin following NEB. In contrast, normal mitotic spindle assembly in higher animal somatic cells occurs through the remodeling of an intact Mt network that does not breakdown at mitosis. Instead the pre-existing Mts are drawn into the two spindle poles as they split/separate prior to NEB. Thus, Mt organization may play a central role in the establishment of spindle polarity in the absence of a true centrosome. To examine this, >100 BSC-1atubGFP cells were cut with a microneedle between nucleus and centrosome generating karyoplasts (acentrosomal, nucleated cells), which were followed by time-lapse microscopy and spindle assembly analyzed. Prior to the onset of mitosis, karyoplasts re-form an acentrosomal MTOC that organizes the interphase Mt network into a single focus. In ~60% of karyoplasts, the MTOC splits apart well before NEB and a bipolar spindle forms; the Mts remain associated with these poles and there are no randomly oriented cytoplasmic microtubules. This splitting requires the activity of Eg5 kinesin. In ~40% of karyoplasts the single interphase MTOC assembles a monopolar spindle that persists following chromosome condensation and NEB - the chromosomes cannot re-sort the Mt array. Monopolar karyoplasts delay in mitosis, but eventually initiate cytokinesis, albeit with multiple furrows, which eventually retract, and the cell exits mitosis as a single daughter. Thus, in karyoplasts with an intact microtubule network - unlike systems with random Mt organization - spindle polarity is determined prior to NEB and is not influenced by chromosomal factors. Importantly, in the absence of centrioles, the ability of the Mt network to bipolarize is severely compromised, leading to an increase in the number of persistent monopolar spindles and failure of cytokinesis. Thus, we conclude that a major role of the centriole-containing centrosome in animal cells is to ensure that strict bipolar spindle assembly is coordinated with entry into mitosis.

2674/B385

Frizzled Pathway and Mitotic Spindle Orientation.

M. Segalen[1], C. A. Martin[1], B. A. Micheau[1], F. M. Rosa[2], N. B. David[2], Y. Bellaiche[1]; [1]UMR144, Institut Curie, Paris, France, [2]U784, ENS, Paris, France

In both vertebrates and invertebrates, the non-canonical Wnt/Frizzled (Fz) pathway plays a major role in the control of mitotic spindle orientation to regulate tissue morphogenesis during symmetric cell division and cell fate specification during asymmetric cell division. Fz controls the orientation of the *Drosophila* asymmetric pI cell (sensory organ precursor) division and thereby specifies the identity of the pI daughter cells. The mechanisms by which Fz regulates mitotic spindle orientation remains to be elucidated. Fz is a seven transmembrane receptor and has been shown to activate non-canonical Wnt signalling via heterotrimeric G proteins. Surprisingly, our analysis reveals that neither of the heterotrimeric G protein subunits Galphao, Galphai and Gbetagamma are required downstream of Fz to control mitotic spindle orientation. However, NuMA, known as Mud in *Drosophila*, is necessary for mitotic spindle orientation downstream of Fz. We are currently studying the molecular mechanisms by which Fz activates NuMA. Furthermore we are analyzing whether the role of NuMA as a downstream effector of the non-canonical Wnt/Fz pathway is conserved in vertebrates.

2675/B386

Phosphatase Control of the Mitotic Spindle Checkpoint.

M. H. Schmitz[1,2], M. Held[1,2], D. Gerlich[1]; [1]Institute of Biochemistry, ETH Zurich, Zurich, Switzerland, [2]Life Science Zurich Graduate School, PhD Programme in Molecular Life Sciences, University of Zurich, Zurich, Switzerland

The progression through mitosis depends on tightly regulated phosphorylation. Several kinases have been previously identified as key mitotic regulators, but if and how counteracting phosphatases contribute to mitotic regulation is largely unknown. Here, we systematically addressed mitotic functions of phosphatases by high-resolution time-lapse imaging and RNAi. To assay mitotic progression in live cells, we stably co-expressed the chromatin marker histone 2B tagged with mRFP (to stage mitotic phases), and a nuclear import substrate tagged with EGFP (to detect nuclear envelope breakdown and re-assembly). Time-lapse movies were automatically recorded from 678 distinct RNAi depletions targeting a genome-wide set of 226 annotated phosphatases. Mitotic timing of more than 100'000 mitotic events was computationally

analyzed by in-house developed software. A large number of RNAi depletions led to delayed mitotic progression. More interestingly, we found that depletion of 16 distinct phosphatases led to significantly accelerated timing from nuclear envelope breakdown until anaphase onset. Because this phenotype has been previously reported for the depletion of spindle checkpoint components Mad2 and BubR1, we next re-screened the entire phosphatase set for spindle checkpoint function in live cells after Taxol treatment. This led to the identification of 17 phosphatases required for spindle checkpoint function, comprising 10 of the 16 phosphatases whose depletion accelerated mitotic progression. Ongoing detailed characterization of these phosphatases showed that depletion of some phosphatases prevented Mad2 localization to kinetochores, while others reduced the overall expression levels of Mad2. We are currently implementing assays to biochemically probe for Mad2-Mad1 interaction after depletion of the phosphatase hits, and the phosphorylation status of known spindle checkpoint components. In conclusion, we identified a set of phosphatases essential for mitotic checkpoint function and timely mitotic progression. It will be interesting to investigate how precisely these phosphatases regulate the known spindle checkpoint components and how their activity is coordinated with mitotic kinases.

2676/B387

Investigation of Mitotic Phosphorylation of CK2α in the Spindle Assembly Checkpoint and Induction of Mitotic Catastrophe.

N. St-Denis, D. R. Derksen, D. W. Litchfield; Biochemistry, University of Western Ontario, London, ON, Canada

Proper mitotic progression is crucial to maintenance of genomic integrity in proliferating cells, and is regulated through an intricate series of events, including protein phosphorylation governed by a complex network of protein kinases. One of the kinases implicated in the regulation of mitotic progession is protein kinase CK2, a small family of enzymes that is overexpressed in cancer and induces transformation in mice and cultured fibroblasts. CK2α, one isoform of the catalytic subunits of CK2, is maximally phosphorylated at four sites in nocodazole-treated cells. To investigate the effects of its phosphorylation on mitotic progression, we generated phosphospecific antibodies against its mitotic phosphorylation sites. In U2OS cells released from S phase arrest, these antibodies reveal that CK2α is most highly phosphorylated in prophase and metaphase. Phosphorylation gradually decreases during anaphase and becomes undetectable during telophase and cytokinesis. Stable expression of phosphomimetic CK2α (CK2α-4D, CK2α-4E) results in aberrant centrosome amplification and chromosomal segregation defects, and loss of mitotic cells through mitotic catastrophe. Conversely, cells expressing nonphosphorylatable CK2α (CK2α-4A) show decreased ability to arrest in mitosis following nocodazole treatment, suggesting involvement in the spindle assembly checkpoint. Collectively, these studies indicate that reversible phosphorylation of CK2α requires precise regulation to allow proper mitotic progression.

2677/B388

The Influence of Catalysis on Mad2 Activation Dynamics.

M. Simonetta[1], R. Manzoni[2], M. Mapelli[1], L. Massimiliano[1], A. Musacchio[1], A. Ciliberto[2]; [1]Dept. of Experimental Oncology, European Institute of Oncology, Milan, Italy, [2]FIRC Institute of Molecular Oncology, Milan, Italy

Mad2 is a key component of the spindle assembly checkpoint, a safety device ensuring faithful sister chromatid separation in mitosis. The target of Mad2 is Cdc20, an activator of the anaphase promoting complex/cyclosome (APC/C). Mad2 binding to Cdc20 is a complex reaction that entails the conformational conversion of Mad2 from an open (O-Mad2) to a closed (C-Mad2) conformer. Previously, it has been hypothesized that the conversion of O-Mad2 is accelerated by its conformational dimerization with C-Mad2. This hypothesis, known as the Mad2-template hypothesis, is based on the unproven assumption that the natural conversion of O-Mad2 required to bind Cdc20 is slow. To address this hypothesis, we developed a real time *in vitro* assay based on the binding of Alexafluor-488-labeled Mad2 to a surface containing immobilized Cdc20 in a flow cell. Using this assay, we gained evidence that the uncatalysed binding of O-Mad2 to Cdc20 is very slow. We demonstrated that conformational dimerization of Mad2 accelerates the rate of Mad2 binding to Cdc20. We show that the binding to Cdc20 is further accelerated by the presence of preformed C-Mad2. Conversely, the rate of binding is reduced to the basal rate in the presence of $p31^{comet}$, a protein that competes with Mad2 dimerization. On the basis of our kinetic measurements, we developed a set of rate equations that deliver excellent predictions of experimental binding curves under a variety of different conditions. Our results strongly suggest that the interaction of Mad2 with Cdc20 is rate limiting for activation of the spindle checkpoint. Conformational dimerization of Mad2 is essential to accelerate Cdc20 binding, but it does not modify the equilibrium of the Mad2:Cdc20 interaction, i.e. it is purely catalytic. These results surpass previously formulated objections to the Mad2-template model and predict that the release of Mad2 from Cdc20 is an energy-driven process.

2678/B389

The Spindle Checkpoint Requires Stabilization of Mad3p by Phosphorylation at Mitosis.

Y. Cheng[2], R. Chen[1]; [1]Institute of Molecular Biology, Academia Sinica, Taipei, Taiwan, [2]Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, Taiwan

The spindle checkpoint delays anaphase onset when any kinetochore is not properly attached to spindle microtubules. The unattached kinetochore recruits spindle checkpoint proteins and promotes the formation of an inhibitory complex composed of Mad2p, Mad3p (BubR1 in metazoans), and Bub3p. This complex sequesters and inhibits Cdc20p, a substrate adaptor for the E3 ubiquitin-protein ligase Anaphase-Promoting Complex (APC), thereby preventing anaphase onset. We herein show that spindle checkpoint protein Mad3p in Saccharomyces cerevisiae is an unstable protein during the cell cycle. The protein is partially stabilized at mitosis independently of the spindle checkpoint and APC. Mutations at five putative Cdc28p phosphorylation sites (mad3-A2) reduce phosphorylation and destabilize Mad3p at mitosis, indicating that phosphorylation is required for the stabilization of Mad3p at mitosis. Furthermore, mad3-A2 cells cannot arrest in the cell cycle in response to spindle disruption. The defective spindle checkpoint is due to the reduced level of Mad3p-A2, as increasing the copy numbers of mad3-A2 gene restores the spindle checkpoint. Our study demonstrates that the spindle checkpoint requires a sufficient level of Mad3p that is maintained by phosphorylation and stabilization at mitosis.

2679/B390

Mitotic Control by the Spindle Matrix.

M. Lince-Faria[1], S. Maffini[1], K. Johansen[3], J. Johansen[3], H. Maiato[1,2]; [1]Intituto de Biologia Molecular e Celular do Porto, Porto, Portugal, [2]Lab Cell and Molecular Biology, Fac de Medicina, Universidade do Porto, Porto, Portugal, [3]Dept. Biochemistry, Biophysics and Molecular Biology, Iowa State University, Ames, IA

The requirement of a putative spindle matrix has long been hypothesized to drive chromosome segregation during mitosis, but evidence for any mitotic role or its existence has never been demonstrated in living somatic cells. Here we show that Megator, the Drosophila counterpart of the human nuclear and nuclear-pore complex protein Tpr, localizes as a labile nuclear matrix that persists during mitosis in living cells, whose shape and behaviour is modulated by spindle microtubule dynamics. Remarkably, Megator/Tpr is required for mitotic control by recruitment of the spindle-assembly checkpoint protein Mad2 to kinetochores in Drosophila and human cells. We propose a conserved mechanism based in the compartmentalization of Mad2 by a spindle matrix that ensures proper spindle-assembly checkpoint control of mitosis.

2680/B391

The Nuclear Spindle Matrix Complex Protein, Chromator, Interacts with the Motor Protein Ncd and Is Required for Proper Microtubule Spindle Formation and Mitosis in *Drosophila* Neuroblasts.

Y. Ding[1], C. Yao[1], U. Rath[1], L. Zhu[1], M. Lince-Faria[2], H. Maiato[2], J. Girton[1], K. Johansen[1], J. Johansen[1]; [1]Biochemistry, Biophysics & Molecular Biology, Iowa State University, Ames, IA, [2]Molecular and Cellular Biology, University of Porto, Porto, Portugal

The concept of a spindle matrix has long been proposed based on theoretical considerations of the requirements for force production to help stabilize the microtubule spindle apparatus during mitosis. However, molecular evidence corroborating its existence and its potential interaction with microtubules and/or motor proteins has been elusive. In *Drosophila* we have identified four nuclear derived spindle matrix proteins Chromator, Megator, Skeletor, and EAST that interact with each other and that redistribute during prophase to form a fusiform spindle structure that persists in the absence of polymerized tubulin. To further study spindle matrix function we have recently generated two loss-of-function alleles in the *Chromator* gene. Our data show that neuroblasts from $Chro^{71}/Chro^{612}$ brain squash preparations have severe tubulin spindle and chromosome segregation defects. The tubulin spindles at metaphase are rudimentary, unfocused, and/or without clear spindle poles. At anaphase chromosomes are lagging and scattered indicating impaired spindle function. This phenotype is similar to that obtained by Chromator RNAi depletion in live S2 cells. A Chromator rescue construct under endogenous promoter control restored all aspects of the mutant phenotype including viability and microtubule spindle morphology. We further show that the C-terminal part of Chromator, but not the N-terminal part, is sufficient to rescue mitotic defects in neuroblasts. In addition, using biochemical and immunoaffinity assays we demonstrate that Chromator and spindle matrix proteins can directly interact with microtubules as well as with the microtubule associated motor protein ncd. The interaction between Chromator and ncd was further supported by genetic interaction assays and the finding that levels of ncd, but not of tubulin, were severely reduced in *Chromator* mutant backgrounds. These findings strongly support the spindle matrix hypothesis and provide evidence for a functional interaction between spindle matrix proteins and the microtubule associated spindle apparatus during mitosis. Supported by NSF grant MCB0817107.

2681/B392

Novel Proteins in Mitotic Spindle Assembly and Disassembly.

J. Tegha Dunghu[1], B. Neumann[2], J. Ellenberg[2], O. J. Gruss[1]; [1]ZMBH-DKFZ Alliance, University of Heidelberg, Heidelberg, Germany, [2]MitoCheck Project Group, EMBL, Heidelberg, Germany

Faithful segregation of chromosomes during cell division is a crucial process that ensures genome stability. This is accomplished by microtubules, polar polymers of α/β-tubulin, which assemble into the mitotic spindle as a cell enters mitosis. Assembly of mitotic spindles require a global change in the constitution and activity of the microtubule binding protein array at mitotic onset. Recent work has been focused on deciphering the composition, function and regulation of the mitotic spindle. Thus, to get a better understanding of the mitotic spindle, unknown molecular components that mediate its assembly have to be identified and the inter-play with known components in the formation and spatial-temporal maintenance of this structure. A number of mitotic microtubule associated proteins (MAPs) are nuclear at interphase and contribute to spindle formation and function during mitosis. Using a proteomic approach we selectively identified proteins of this category and unveiled 50 poorly characterized human gene products, among them the echinoderm-microtubule associated protein-like gene product, Eml3. Eml3 belongs to the family of human echinoderm microtubule associated proteins (EMAPs) of which five members have been characterised. EMAP proteins possess a characteristic hydrophobic EMAP-like protein (HELP) domain which is thought to mediate binding to microtubule. Indirect Immunofluorescence shows that Eml3 colocalises with spindle microtubules throughout mitosis. At interphase, Eml3 both colocalises with cytoplasmic microtubules and accumulates in interphase nuclei. Using YFP-fusion constructs of Eml3, we spotted a nuclear localisation signal as well as confirmed the microtubule-binding domain of Eml3. Functional analysis of Eml3 using time-lapse fluorescence microscopy and detailed end-point analysis of phenotypes after siRNA knockdown demonstrates an important role of Eml3 in proper metaphase chromosome alignment. Our proteomic identification screen combined with sensitive phenotypic analysis therefore provides a reliable platform for the identification and characterisation of proteins important for proper cell division.

2682/B393

In Silico Reconstitution of Spindle Assembly: Speed and Error of Search and Capture.

R. Paul[1], R. Wollman[2], A. Mogilner[1]; [1]Dept. of Neurobiology, Physiology and Behavior, University of California Davis, Davis, CA, [2]Department of Chemical and Systems Biology, Stanford University, Stanford, CA

During mitosis, in prometaphase, a bipolar spindle, in which microtubules connect centrosomes at the 'poles' to chromosomes at the 'equator' self-assembles. Whether this assembly goes through centrosomal (dynamically unstable microtubules search in space until kinetochores are captured) or chromosomal (microtubules grow from chromosomes and get focused to the centrosomes by motors) pathway is still uncertain and seems to be cell type-specific. Furthermore, some evidence suggests that these two pathways are not mutually exclusive. Using extensive numerical simulations, we analyzed the effects of chromosomal volume and movements on the speed and accuracy of the spindle assembly when these two pathways are combined. Our results suggest that an optimal spindle assembly is achieved when rates of chromosomal

movements are of the same order of magnitude as rates of the microtubule dynamic instability. We further quantify syntelic and merotelic errors, which occur with surprising frequency in the simulations, and discuss how the error numbers depend on geometry and kinetics of the spindle assembly.

Cytokinesis II (2683 – 2705)

2683/B394

IQGAP1 Regulates Cell Proliferation through a Novel CDC42-mTOR/PI3K Pathway.

M. A. Osman; Biotechnology Institute, Cornell University, Ithaca, NY

Cell proliferation requires regulated coordination of cell division and cell growth to ensure constant cell size through the division cycles. IQGAP1, an effector of CDC42 GTPase has been implicated in regulation of cytoskeleton, exocytosis and in human cancers. Here we show that IQGAP1 localizes in the midbody ring during cell abscission and regulates cell proliferation. This activity requires phosphorylation of IQGAP1 and its binding to CDC42, which also localizes to the midbody. Expression of IQGAP1 C-terminus enhanced migration and reduced cell size whereas expression of the N-terminus increased the cell size, and inhibited migration. Further, the N-terminus of IQGAP1 interacts with mTOR and activates Akt and both proteins are required for IQGAP1-mediated cell proliferation. These findings are consistent with a model where IQGAP1 serves as a phosphorylation-sensitive conformation switch to coordinate cell growth with division through a novel CDC42-mTOR/PI3K pathway with implications for cancer therapy.

2684/B395

Phosphoregulation of F-BAR/PCH Family Member Cdc15.

R. Roberts-Galbraith[1], D. M. Clifford[1], M. G. Chambers[2], J. Chen[1], M. D. Ohi[2], K. L. Gould[1]; [1]Howard Hughes Medical Institute and Department of Cell and Developmental Biology, Vanderbilt University, Nashville, TN, [2]Department of Cell and Developmental Biology, Vanderbilt University, Nashville, TN

pombe cdc15 homology (PCH) proteins participate in diverse cellular processes, such as cell morphology, cytokinesis, endocytosis, and motility, by bridging the cell cortex and actin cytoskeleton. Most PCH family members share a conserved domain architecture of an N-terminal F-BAR domain and a C-terminal SH3 domain. Recently, the structure and function of F-BAR domains of this family have been characterized in detail. However, modes of regulation of PCH family members have not been investigated. The founding member of this family, *Schizosaccharomyces pombe* Cdc15, promotes contractile ring assembly and organization and is essential for cytokinesis. As with many F-BAR/PCH proteins, Cdc15 exists as a phosphoprotein *in vivo*, specifically becoming hypophosphorylated during mitosis. Based on this observation, we have investigated dephosphorylation of Cdc15 as a potential regulatory step in contractile ring formation in *S. pombe*. Here, we show that dephosphorylation of purified full-length Cdc15 causes conformational changes and promotes oligomerization that may be key to the scaffolding role of Cdc15 in cytokinesis. Dephosphorylation of Cdc15 also enables it to bind and recruit formin Cdc12, which is also essential for cytokinesis in *S. pombe*. The influence of dephosphorylation on the ability of Cdc15 to bind and deform membranes is also being investigated. We have investigated regulators of Cdc15 phosphorylation and have found that Cdc14-like phosphatase Clp1 dephosphorylates Cdc15 *In Vivo* and *in vitro*. *In Vivo*, elimination of Clp1 activity prevents some Cdc15 dephosphorylation and results in altered Cdc15 dynamics at the contractile ring. Additionally, several kinases phosphorylate Cdc15 *in vitro*, including Septation Initiation Network kinase Sid2 and cyclin-dependent kinase Cdc2, supporting a complex mechanism of regulation for Cdc15. We are currently investigating the role of specific combinations of phosphosites in regulating Cdc15 during the cell cycle. Taken together, our results shed light on a major mode of regulation for the F-BAR/PCH family of proteins.

2685/B396

The Role of Zw10 in *Drosophila* Cytokinesis.

A. Wainman[1], M. Giansanti[1], M. l. Goldberg[2], M. Gatti[1]; [1]Genetics and Molecular Biology, Sapienza, Università di Roma, Rome, Italy, [2]Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY

ZW10, along with the other members of the conserved Rod-Zwilch-Zw10 (RZZ) complex, is required for proper functioning of the spindle assembly checkpoint. It has been also reported that Zw10 is required for spermatocyte cytokinesis, but its role in this process has never been defined. We found that dividing spermatocytes of *zw10* mutants assemble normal central spindles and acto-myosin rings. However, these rings fail to constrict to completion and the central spindles progressively degenerate. The same phenotype has been previously observed in mutants defective in membrane-related functions, such as *giotto (gio), four wheel drive (fwd), four way stop (fws)* and *rab11*. During ana-telophase, Zw10 accumulates across the spindle envelope (SE) mid-zone. In *zwilch* and *rod* mutants, in which the RZZ complex does not associate with the kinetochores, Zw10 was no longer enriched at the SE mid-zone but localized to both the Golgi stacks and the endoplasmic reticulum (ER). These results suggest that an increase in the Zw10 pool not associated with the kinetochores results in an enrichment of this protein at both the Golgi and the ER. In addition, these observations indicate that Zw10 localization at the SE mid-zone is irrelevant for cytokinesis. Although *zw10* mutants did not show obvious defects in the ER and Golgi structure, our results suggest a role for Zw10 in membrane trafficking during cytokinesis.

2686/B397

A Role for Tubulin Acetylation in Cytokinesis.

N. Elia, J. Lippincott-Schwartz; NIH, Bethesda, MD

Microtubules play a key role in cell division and in cytokinesis. Late in telophase, a microtubule based intracellular bridge, the midbody, is the only connection between the two daughter cells. This midbody microtubule structure is required for both initiation and completion of cytokinesis in a mechanism that is not clearly understood. Microtubules are subjected to a series of post translational modification. Among these modification is the acetylation of lysine 40 of α-tubulin. Initial immuno-fluorescence staining has shown that the midbody microtubules

are heavily acetylated. To test the role of tubulin acetylation in cytokinesis of mammalian cells, we generated stable MDCK cell lines over-expressing a GFP tagged version of a K40R α-tubulin mutant that is unable to undergo acetylation. Initial characterization of this cell line revealed a two-fold increase in the number of cells in late telophase; cell that contained a midbody. To better understand the nature of this phenotype, we applied a comparative live cell imaging analysis of cells during cytokinesis. This analysis revealed that the midbody structure is more persistence in the GFP-K40R-tubulin cell line, with a two-fold increase in lifetime compared to GFP-WT-tubulin cell line. Tubulin acetylation thus plays a role in midbody abscission. The mechanistic basis for this function during cytokinesis is under current investigation

2687/B398

A System-Wide of Biochemical Characterization of the Cytokinesis-phase.

N. Ozlu[1,2], F. Monigatti[2], B. Renard[4], C. Field[1], H. Steen[2], T. Mitchison[1], J. J. Steen[3]; [1]Systems Biology, Harvard Medical School, Boston, MA, [2]Department of Pathology, Harvard Medical School and Children's Hospital, Boston, MA, [3]Department of Neurobiology, Harvard Medical School and Children's Hospital, Boston, MA, [4]Interdisciplinary Center for Scientific Computing, University of Heidelberg, Heidelberg, Germany

Cytokinesis is the physical separation of one cell into two at the end of the cell cycle. It is one the shortest phases of the cell cycle displaying dramatic changes in cell morphology. During cytokinesis, the “spindle”, a microtubule based structure, temporally and spatially controls the actomyosin-based contraction of the cortex to ensure cell division between separating chromosomes. Because these changes are modulated by protein-protein interactions and kinase activities, we took a global quantitative proteomic approach to understand the regulation of the cytoskeleton during cytokinesis using SILAC (Stable isotope labeling with amino acids in cell culture). We performed a microtubule sedimentation assay from differentially labeled mitotic and cytokinesis and interphase cells. Using LC/MS on a linear ion trap/orbitrap mass spectrometer (LTQ-Orbitrap), we identified over a thousand of proteins in total. 25 of these proteins more enriched in cytokinesis microtubules as comparing to mitosis and interphase. To understand regulation by protein phosphorylation in cytokinesis, we undertook a global phosphorylation experiment to study the phosphorylation dynamics of all soluble proteins and the proteins associated with microtubules in cells undergoing cytokinesis. This analysis identified over 4500 phosphorylation sites, of which 630 were mitosis specific and 160 were cytokinesis specific phosphorylation sites. An analysis of the phosphorylation consensus sequences revealed that Aurora-B to be one of the active cytokinesis kinases. Focussing on Aurora B, we performed a detailed quantitative interaction study of Aurora-B, which revealed both novel and known interactors of Aurora-B. For example an FH1/FH2 domain containing actin binding protein interacts with Aurora-B and this interaction enhances during cytokinesis. Using proteomics allowed us to study the interactions of all different compartments of cytokinesis at once in a quantitative manner that provided mechanistic insight.

2688/B399

Systematic Analysis of Cytoskeletal Motor Protein Localization and Binding Partners in Hela cells.

Z. Maliga, M. Junqueira, A. Schevchenko, A. A. Hyman; MPI-CBG, Dresden, Germany

Microtubules and actin filaments are polarized, dynamic, self-assembling biopolymers that define orthogonal cytoskeletal networks essential for intracellular transport and cell division. Although some kinesin and myosin motors have well understood roles in mictotubule and actin-based transport or cell division, few of the 45 kinesins and 39 myosins encoded in the human genome are well characterized to date. To provide a better understanding of these proteins, we modified bacterial artificial chromosomes for use as transgenes to monitor protein localization in live cells and to recover interaction partners by co-immunoprecipitation from cell extracts. We generated a total of 23 stably transfected myosin and 41 kinesin Hela-Kyoto cells, then analyzed these by live-cell video microscopy to show proper targeting of well characterized proteins, then to catalogue the interphase localization patterns. Out of 8 recombinant mitotic kinesins, 7 were properly targeted through the cell cycle. In addition, 6 myosins were targeted to the mitotic cortex, of which 2 were enriched at the cleavage furrow. Ten motor transgenes are targeted to recognizable (not nuclear or multi-punctate) interphase compartments: centrosomes, filopodia, lamellipodia, nuclear envelope, mitochondria, or regions of cell-cell contact. In parallel, we are performing co-IP analysis to identify motor protein interactions partners by anti-GFP immunoprecipitation and FT-tandem mass spectrometry. Of 38 transgenic cell lines analyzed so date, 29 have recovered the motor transgene and a total of 60 bait-specific candidate interaction partners. Further, we illustrate a candidate validation pipeline (BAC transgenesis RNAi, chemical inhibitors, co-IP, and live-cell imaging) to understand the regulation of MKLP1 targeting and activity in cytokinesis by PLK-1. Our study provides a comprehensive starting point to study most myosin and kinesin motor proteins and to refine our understanding of sub-cellular compartmentalization.

2689/B400

A Screen to Identify Novel Cytoskeletal Regulators.

R. Green[1], A. Audhya[2], K. Oegema[1], A. B. Desai[1]; [1]Cellular and Molecular Medicine, Ludwig Institute for Cancer Research, UCSD, La Jolla, CA, [2]Department of Biomolecular Chemistry, University of Wisconsin-Madison Medical School, Madison, WI

The *C. elegans* germline and early embryo has emerged as an important system for the systematic characterization of genes that regulate the cytoskeleton, including genes required for centrosome regulation, the microtubule-based processes of nuclear positioning and spindle assembly, and actin-based processes such as polarity establishment and cytokinesis. Large scale RNAi screens with *C. elegans* embryos have identified the complete set of genes required for early embryonic divisions using high-content DIC microscopy. However, it is likely that many proteins that regulate the cytoskeleton may have been missed by this approach due to a prior requirement in maintenance of the syncytial gonad and/or in the cytokinesis-like process of oocyte formation. Thus, the collection of 562 genes whose inhibition results in sterile animals represents a substantial untapped resource for the identification of novel cytoskeletal regulators. To characterize this collection, we performed an RNAi screen in which gonad structure was monitored in a strain co-expressing GFP:PH and RFP:Histone, to label the plasma membrane and the chromosomes. Imaging data was annotated for 92 possible defects and clustering analysis grouped the phenotypes into ~25 broad classes that could be further partitioned into groups representing specific phenotypes. This data will be made available to the scientific community in a searchable online format upon publication. Known cytoskeleton-related proteins, such as actin, α-tubulin, dynein, and Rho, all give rise to a multi-nucleated gonad following RNAi. Approximately 30 novel genes also fell into this class,

including several that are strong phenotypic matches to known cytoskeleton-related proteins. These unknown genes are likely to include novel interactors of the cytoskeleton. Supporting this idea, a GFP-fusion of unknown SS193 co-localizes with myosin in the gonad and localizes to the contractile ring in dividing embryos, suggesting this novel protein may be required for cell division. This screen complements previous characterizations of the embryonic lethal collection to provide systematic information on the entire collection of *C. elegans* genes required for regulation of the cytoskeleton.

2690/B401

Focal Adhesion Kinase (FAK) Mediates Blastomere Abscission and Vesicle Trafficking during Early Embryonic Cleavage in the Sea Urchin Embryo.

M. B. Hille[1], B. Schumpert[1], M. G. García[1], G. Wessel[2]; [1]Biology, University of Washington, Seattle, WA, [2]Department of Molecular and Cellular Biology and Biochemistry, Brown University, Providence, RI

We found that focal adhesion kinase (FAK) is expressed during early cleavage divisions of the sea urchin embryo. FAK, as detected by a polyclonal antibody to the *Lytechinus variegatus* protein, is nearly absent in the egg and zygote, and then oscillates in abundance during the first cleavages after fertilization. Such cycling is consistent with the occurrence of a destruction box in the N-terminal sequence of *L. variegatus* FAK. During cleavage, FAK is found in the cytoplasm and enriched in the cortex, but is excluded from the cleavage furrow. To investigate whether FAK has a role in membrane events during early cleavage, we interfered with FAK function by microinjecting *L. variegatus* eggs with the anti-FAK antibody or with FAK antisense morpholino oligonucleotides. Both treatments led to regression of the cleavage furrow immediately before abscission between daughter blastomeres. Nothing is known about the role of FAK in cleavage. Abscission requires regulation of actin microfilaments, rho inactivation, the disassembly of the contractile ring, and vesicle delivery to a stable central spindlin with an exocyst complex (Simon et al, 2008, Schweitzer and Souza-Schorey, 2004). FAK binds both Rho GEFs and Rho GAPs (Tilghman and Parsons, 2008, and thus, may mediate the turnover of Rho and actin microfilaments, and the final disassembly of the contractile ring. We showed that FAK localizes to the cortex and actin structures, including the region of the membrane that follows the cleavage furrow as it ingresses. FAK may also interact with the central spindlin as a scaffolding protein to stabilize the exocyst complex for vesicle fusion. FAK knockdown with morpholino oligonucleotides or antibodies also resulted in an accumulation of endocytic vesicles. Vesicle trafficking is known to be required for abscission to occur (Barr and Grunberg, 2007). One possibility is that FAK-src interactions could be required for the dynamics of this vesicle trafficking (Sandilands and Frame, 2008). In summary, we conclude that FAK is necessary for successful cleavage and is required for blastomere abscission and the dynamics of vesicle trafficking. These results are the first to document a functional role for FAK during embryonic cleavage.

2691/B402

Meiosis-Specific Movement of the Contractile Ring during Polar Body Formation in *Caenorhabditis Elegans.*

A. S. Fabritius, F. McNally; Cellular and Molecular Biology, University of California Davis, Davis, CA

During *C. elegans* female meiosis, spindle rotation and shortening produce a very short spindle contacting the cortex. The small size and extreme asymmetric positioning of the anaphase spindle may minimize polar body volume, thus maximizing egg volume. Consistent with this hypothesis, partial loss of function in the microtubule-severing protein MEI-2 produces abnormally large spindles and abnormally large polar bodies. To determine if polar body formation is controlled directly by spindle length, we used time-lapse imaging of wild-type embryos to determine whether the meiotic contractile ring bisects short spindles or if the ring contracts asymmetrically at the cortical end of the spindle. Bisection of the spindle would indicate that spindle length is a determinant in polar body size. Time-lapse imaging of GFP-NMY-2 and GFP-MOE reveals that the meiotic contractile ring moves in two dimensions. The ring contracts and at the same time pulls away from the eggshell. Time-lapse imaging of GFP-tubulin and GFP-histone reveals that one pole of the anaphase spindle remains stationary relative to the eggshell at the time the ring moves inward down the length of the spindle. We are currently imaging strains expressing GFP-tubulin and GFP-NMY-2 as well as GFP-NMY-2 and mCherry-histone to precisely determine how far down the length of the spindle the ring moves as it pulls away from the eggshell. Preliminary time-lapse images suggest that the ring bisects the meiotic spindle during anaphase B. In addition, we plan to use RNAi to dissect the mechanism by which the ring moves down the length of the spindle.

2692/B403

Cleavage Furrow and Cortex Stability during Cytokinesis.

J. Sedzinski[1, 2], A. Oswald[1], M. Biro[1], E. Paluch[1, 2]; [1]MPI-CBG, Dresden, Germany, [2]IIMCB, Warsaw, Poland

Cytokinesis relies on a tight spatial and temporal control of cortical contractility. Starting from anaphase, the cortical network reorganizes and gives rise to the cytokinetic ring, which separates the two daughter cells. Strikingly, cleavage does not only result from localized constriction of the ring but requires global coordination of cortical activities. In order to understand the role of mechanical properties of the cortex during cytokinesis, we employed two techniques to locally perturb the cortex: laser ablation and local delivery of drugs known to affect the actin cytoskeleton. We found that these local cortex perturbations lead to cleavage furrow oscillations where the latter moves back and forth over the cell surface. This striking behavior is preceded by several cortex depolymerization and reassembly events. The cell cortex reassembles in a non-symmetrical manner, which causes an unbalanced contractile force distribution between the two parts of the dividing cell. The more contractile part squeezes cytoplasm into the less contractile part, which initiates the oscillations. Strikingly, the analysis of control divisions with particle image velocimetry showed that furrow positioning is inherently unstable and exhibits small fluctuations about the cleavage plane. However, despite these fluctuations, the furrow is normally maintained in the center plane of the cell during cytokinesis. We propose that the formation of blebs can maintain cytokinetic stability by locally releasing tension in contractile parts of the cell. This prevents asymmetric build-up of contractile forces within the cortex and thus steadies furrow positioning at the center of the cell.

2693/B404

Altering Mitotic Apparatus Polarity and Cell Geometry Reveals Multiple Roles for Microtubules in Furrow Specification.

D. Rodriguez[1,2], V. Foe[2], C. Shuster[1,2]; [1]Dept of Biology, New Mexico State University, Las Cruces, NM, [2]Center for Cell Dynamics, Friday Harbor Laboratories, Friday Harbor, WA

Cytokinesis in animal cells entails the formation of a transient zone of localized cortical contractility, whose position is determined by the mitotic apparatus. Two mechanisms have been proposed to explain the relationship between the mitotic apparatus and cleavage plane formation in large embryonic cells: 1) polar relaxation, whereby an inhibitory signal associated with astral microtubules restricts contractility to the equator where microtubule density is presumed to be low; 2) equatorial stimulation, whereby a stimulatory signal is delivered to the cortex where astral microtubules from the two poles overlap. To determine whether either or both mechanisms explain furrow specification in echinoderm zygotes, we manipulated cell geometry and spindle polarity, and either imaged cells live or fixed and processed manipulated cells to reveal microtubules and Pser19RLC-phosphorylated myosin (Pser19RLC). To restrict the area of cortex influenced by the spindle, cells were reshaped into long cylinders. Elongated telophase cells with bipolar spindles formed cleavage furrows at the midpoint between spindle poles, and displayed elevated levels of cortical Pser19RLC at the cleavage plane (where stable microtubules impinge and a normal furrow formed), a slight Pser19RLC depression near asters (composed predominantly of dynamic microtubules), and intermediate Pser19RLC levels at the distal pole (devoid of microtubules). Cylindrical telophase cells with monopolar spindles exhibited random contractility (and elevated Pser19RLC) distal to the mitotic apparatus where astral microtubules are absent. Monopolar cells treated with hexylene glycol (which stabilizes microtubules) suppressed these ectopic contractions and enhanced furrowing adjacent to the spindle. Our experiments suggest that during cytokinesis the zone of cortical contractility is determined both by a stimulatory signal from the spindle midzone (conveyed more effectively by stable than dynamic microtubules) and by a relaxing signal associated with dynamic astral microtubules. In bipolar cells, the reach of the stimulatory signal is confined by the presence of the flanking inhibitory signals from the asters, whereas in monopolar cells this confinement does not occur.

2694/B405

The Human Nuclear Pore Protein Nup153 Affects Cytokinesis via the Spindle Assembly Check Protein Mad1.

B. Fahrenkrog[1], D. K. Shumaker[2], Y. Lussi[1], T. Shimi[2], R. Goldman[2]; [1]M.E. Mueller Institute for Structural Biology, Biozentrum, Basel, Switzerland, [2]Feinberg School of Medicine, Department of Cell and Molecular Biology, Northwestern University, Chicago, IL

Nuclear pore complexes (NPCs) present the exclusive sites for macromolecular trafficking between the cytoplasm and nucleus of eukaryotic cells. Residing in the double membrane of the nuclear envelope (NE), they are composed of about 30 different nucleoporins, one of which is Nup153. Based on its amino acid sequence, Nup153 consists of an N-terminal domain required for its targeting to the NE and NPC; a central zinc-finger domain critical for NE breakdown at the onset of mitosis; and a C-terminal domain housing multiple phenylalanine-glycine repeat motifs that provide interaction sites for a number of nuclear transport receptors due to which Nup153 functions in nuclear import and export. Here we show that the interaction of Nup153 with the spindle assembly checkpoint (SAC) protein Mad1 is important for the correct timing of cytokinesis. Over-expression of human Nup153 in HeLa cells leads to the appearance of multinucleated cells including micronuclei as analysed by confocal laser scanning microscopy. Expression of GFP-Nup153 induces the formation of multipolar spindles that coincide with supernumerary centrosomes and unresolved cytokinesis. Whereas cell cycle progression remains unaffected, expression of Nup153 causes a strong increase in the number of aneuploid cells as revealed by flow cytometric analysis of the DNA content in these cells. Furthermore, knocking down Nup153 by using RNA interference demonstrates a delay in cytokinesis along with an increased number of midbodies in HeLa cells. Moreover, the depletion of Nup153 coincided with a loss of Mad1 from the NPC and during metaphase Nup153 and Mad1 appear associated at centromeres. Immunoprecipitation and solution binding assays revealed that Mad1 binds directly to the NPC targeting region of Nup153 in its N-terminus. Our data suggests that Nup153 affects timing of cytokinesis independent of its ability to interact with soluble transport receptors, but by regulating the localization of Mad1, most likely during the metaphase/anaphase transition.

2695/B406

Constriction of the Contractile Ring in Fission Yeast Protoplasts.

B. Guirao[1], M. Stachowiak[1], P. G. Rodriguez[2], T. Pollard[2], B. O'Shaughnessy[1]; [1]Department of Chemical Engineering, Columbia University, New York, NY, [2]Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT

Cytokinesis is the final stage of eukaryotic cell division where constriction of a contractile ring helps divide the cell into two daughters. In fission yeast ring components include actin, myosin-II, formin Cdc12p and alpha-actinin Ain1p. How components are spatiotemporally organized into a contractile machine is not established. Quantitative modeling can help determine organization and mechanisms by testing arrangements. A complication is that constriction occurs concomitantly with septum deposition between the daughters. To separate these coupled processes we studied ring constriction in fission yeast protoplasts expressing Rlc1p-tdtomato whose walls were digested enzymatically. Treated cells are rounded (diameter 2.5-5 μM) and some assembled a complete ring. On enzyme removal but before the cell surface stained with Calcofluor, assembled rings constricted over 50-200 min (cf. ~30 min for normal cells) while sliding toward one pole, suggesting the ring is tensile and constriction occurs without septation. Using mathematical modeling we tested different proposed ring structures by comparing predictions using physiological parameter values to experimentally measured time profiles of ring radius, R(t). The model describes constriction in terms of forces from myosins and internal and external drag which may originate from ring-cytoplasm, ring-membrane and ring-cell wall interactions. We implemented the model assuming a sarcomeric structure similar to that of striated muscle and stress fibers. The R(t) profiles were fit using a value for the external drag coefficient ~15-50 times that of all ~63 nodes from which the ring is initially condensed (Vavylonis et al, Science, v319, p97, 2008). This large drag could originate from connections between the ring and partially reformed cell wall. When a more random less sarcomeric arrangement was implemented the model predicted diminished tension, and fitting R(t) profiles required external drag coefficients diminished by a factor depending on the degree of randomness. Measured protoplast constriction kinetics are consistent with (i) sarcomeric ring arrangement and strong external drag coupling or (ii) random arrangement and weaker drag coupling.

2696/B407

Cell Cycle Regulated Phosphorylation and Localization of Nuf, a Rab11 Effector, May Drive Furrow Formation in the Drosophila

Embryo.

J. Crest, A. Royou, W. Sullivan; MCD Biology, University of California, Santa Cruz, CA

Animal cytokinesis involves both actin-myosin based contraction and vesicle mediated membrane addition. In many cell types, including early Drosophila embryos, Nuf/FIP3, a Rab11 effector, mediates recycling endosome-based vesicle delivery to the cytokinesis furrows. Nuf exhibits dramatic cell cycle dependent changes in its phosphorylation state that are correlated with its association with Rab11 at the recycling endosome (RE). These observations raise a number of issues concerning the relationship between Nuf localization and phosphorylation state and the specific kinases involved. Preliminary work suggests Nuf phosphorylation may prevent it from localizing to the centrosome-associated RE. We have also accumulated genetic, biochemical and cellular data suggesting Polo kinase targets Nuf. These studies lead us to suggest that Polo-mediated phosphorylation of Nuf may prevent its Dynein-based transport to and interaction with Rab11 at the RE. We propose that this cell cycle mediated phosphorylation of Nuf may control the timing of furrow ingression and regression in the Drosophila embryo.

2697/B408

Vesicle-Mediated Delivery from the RE Promotes Both Cortical Actin and Membrane Integrity during Ingression of the Cytokinetic Furrow.

J. Cao[1], R. Albertson[1], B. Riggs[1], L. Serbus[1], C. Field[2], W. Sullivan[1]; [1]MCD Biology, UCSC, Santa Cruz, CA, [2]Systems Biology, Harvard Medical School, Boston, MA

Vesicle-based membrane delivery from the recycling endosome (RE) has proven to be a key element in the formation of the cytokinesis furrow. A puzzling aspect of this process is that disruptions in the RE produce pronounced defects in cortical actin organization as well as membrane organization at the furrow. For example in the *Drosophila* early embryo, mutants in Nuf (Nuclear-fallout), a Rab11 effector that is essential for RE function, produce defects in F-actin and membrane integrity during furrow ingression. To understand the connection between actin and membrane integrity, we relied on live analysis of furrow formation. We discovered that in *nuf* mutant embryos, an initial loss of F-actin at the furrow is followed by loss of the associated furrow membrane. Wild-type embryos treated with Latrunculin A or Rho inhibitor display similar defects as *nuf* mutant embryos or embryos over-expressing Cofilin, an actin depolymerizer. Significantly, drug- or Rho-GTP-induced increase of actin polymerization or genetically mediated decrease of actin depolymerization suppresses the *nuf* mutant F-actin and membrane defects. In addition, RhoGEF2 does not properly localize at the furrow in *nuf* mutant embryos and that RhoGEF2-Rho1 pathway components show strong specific genetic interactions with Nuf. We propose a model in which RE-derived vesicles promote furrow integrity by regulating the rate of actin polymerization through the RhoGEF2-Rho1 pathway. Finally, we observed strong genetic interactions between Nuf and actin remodelers in other cell types as well.

2698/B409

A Role for Very-Long-Chain Fatty Acids in Furrow Ingression during Cytokinesis in *Drosophila* Spermatocytes.

I. Szafer-Glusman[1], M. Giansanti[3], R. Nishihama[2], J. Pringle[2], M. Gatti[3], M. Fuller[1,2]; [1]Developmental Biology, Stanford School of Medicine, Stanford, CA, [2]Genetics, Stanford School of Medicine, Stanford, CA, [3]Dipartimento di Genetica e Biologia Molecolare, Università di Roma "La Sapienza", Roma, Italy

Cell shape and membrane remodeling rely on regulated interactions between the lipid bilayer and cytoskeletal arrays at the cell cortex. During cytokinesis, animal cells build an actomyosin ring anchored to the plasma membrane at the equatorial cortex. Ring constriction coupled to plasma membrane ingression separates the two daughter cells. Plasma-membrane lipids influence membrane biophysical properties such as membrane curvature and elasticity and play an active role in cell function, and specialized membrane domains are emerging as important factors in regulating assembly and rearrangement of the cytoskeleton. Using live imaging, we now show that mutations in the gene *bond*, which encodes a *Drosophila* member of the family of Elovl proteins that mediate elongation of very-long-chain fatty acids, block or dramatically slow cleavage-furrow ingression during early telophase in dividing spermatocytes. In *bond*-mutant cells, the contractile ring frequently detaches from the cortex and constricts or collapses to one side of the cell, and the cleavage furrow regresses. Our findings implicate very-long-chain fatty acids or their derivative complex lipids in allowing supple membrane deformation and the stable connection of cortical contractile components to the plasma membrane during cell division.

2699/B410

Binding of the Phospholipid PIP2 to Yeast Septin Proteins Is Required for Cell Division.

V. Votin, T. Allyn, J. Thorner; University of California, Berkeley, CA

Septins constitute a conserved family of proteins that constitute part of the cortical cytoskeleton and are involved in polarized growth, cytokinesis, and other processes in which membrane remodeling occurs. Cytokinesis in Saccharomyces cerevisiae requires a collar of septins at the mother-bud neck to serve as a protein scaffold and diffusion barrier. Septins localize immediately adjacent to the plasma membrane at the bud neck. This study seeks to determine how septins are recruited or anchored to the plasma membrane, specifically to the bud neck. Phosphatidylinositol-4,5-bisphosphate (PIP2) is a good candidate for septin recruitment, because PIP2 is enriched at the site of cytokinesis and is required for cytokinesis in mammalian cells (Emoto et al, J Biol Chem, 2005) and for proper septin localization in yeast (this study and Rodriguez-Escudero et al, Biochem J, 2005). We have observed a PIP2 marker at the yeast bud neck. Liposome flotation assays show that the yeast septins Cdc3, Cdc11 and Cdc12 bind PIP2 in vitro. We identified the basic residues of Cdc3 that contribute to PIP2-binding. Moreover, the physiological importance of PIP2-binding is indicated by effects of Cdc3 basic-region mutants on normal cytokinesis and bud morphology in vivo. Therefore, direct PIP2-septin interaction at the bud neck is required for cytokinesis.

2700/B411

Deletion of Myosin-II Gene Enables Divergent Evolution of Alternative Pathways for Cytokinesis through Aneuploidization Events.

G. Rancati, N. Pavelka, B. Fleharty, A. Noll, R. Allen, K. Walton, A. Perera, K. Staehling-Hampton, C. Seidel, R. Li; Stowers Institute for Medical Research, Kansas City, MO

A hallmark of biological systems is their evolvability, the capacity to adjust to perturbations through evolutionary processes. In this work, we used a unique experimental design to inquire into the evolvability of yeast cells in response to the deletion of a gene encoding a major structural protein important for cell division. Yeast cells deleted of the *MYO1* gene, encoding the only myosin-II motor protein, initially exhibited severe growth and cytokinesis defects but were able to rapidly evolve alternative pathways to restore both processes. A combination of electron microscopy and live cell imaging revealed the emergence of at least three major mechanisms to achieve closure of the mother-bud junction, leading to formation of septa with distinct morphologies. Transcriptome profiling indicated that the evolved cytokinesis mechanisms correlated with and relied upon gene expression changes in specific pathways with no obvious direct relationship to Myo1-dependent functions. We then determined the genetic changes that occurred in the *myo1Δ* e-strains using an array of genome analysis methods. The results revealed a strong correlation between favorable evolutionary outcome and polyploidization and aneuploidization. Aneuploidy could account for not only gene expression changes quantitatively correlated with chromosome stoichiometries but also changes far beyond this effect. We show that changes in gene copy numbers of specific regulatory molecules, which mimic the effect of aneuploidy, can recapitulate one of the evolved cytokinesis phenotype in *myo1Δ* cells. These results suggest that even conserved core processes can be highly evolvable and provide insights into the relationship between evolvability and the complexity that characterize cellular systems.

2701/B412

Rate-Limiting Role for Cell-Wall Synthesis, Not Actomyosin-Ring Contraction, in Cleavage-Furrow Ingression during *Saccharomyces Cerevisiae* Cytokinesis.

R. Nishihama, J. Pringle; Department of Genetics, Stanford University School of Medicine, Stanford, CA

It is widely believed that the contraction of an actomyosin ring results in the membrane invagination that forms the cleavage furrow during cytokinesis in animal and fungal cells. In the budding yeast *Saccharomyces cerevisiae*, the contractile ring functions in conjunction with the formation of a chitinous primary septum (PS) by the integral-membrane protein chitin synthase 2 (Chs2p). Deeletion of *MYO1* (the only myosin II gene) blocks actomyosin-ring (AMR) formation but is nonetheless nonlethal. Electron microscopy of *myo1Δ* cells revealed that PS-like, chitin-containing septal structures could still form but were typically misoriented, suggesting that the AMR is unnecessary for membrane invagination and instead only guides its direction in this organism. In contrast, deletion of *CHS2* causes complete loss of PS-like membrane-invagination structures [instead, broad invagination occurs due to massive deposition of secondary septum (SS)-like material] and detachment of the AMR from the membrane followed by abrupt contraction to one side, indicating that coordination of AMR contraction with membrane invagination requires Chs2p. To explore the coordination mechanisms further, we examined the effects of various reduced-activity mutations of *CHS2* on AMR contraction and septum formation. Point mutations that completely impair chitin-synthase activity caused phenotypes identical to those caused by *chs2Δ*, suggesting that there is no structural role for Chs2p in stabilizing the AMR. Mutations that reduce Chs2p activity ~6-fold slowed AMR contraction and caused simultaneous formation of PS and SS, suggesting slowed PS formation and premature SS formation (normally, SS formation begins only after completion of PS formation). Thus, in *S. cerevisiae*, the rate of cleavage-furrow ingression is not determined by the contraction force generated by the AMR but instead by Chs2p-mediated chitin synthesis, which may support the invagination created by membrane-remodeling proteins.

2702/B413

Regulation of Fission Yeast Myosin-II by the UCS Protein and Tropomyosin.

M. Lord, B. Stark, T. E. Sladewski; Molecular Physiology & Biophysics, University of Vermont, Burlington, VT

Fission yeast myosin-II (Myo2p) is required for assembly and constriction of the contractile ring during cytokinesis. We employed in vitro approaches with Myo2p to examine molecular mechanisms regulating its motor activity. UCS (Unc-45-/Cro1p-/She4p-related) proteins are thought to be involved in the folding and stabilization of myosin motors in cells. Our previous work has shown that the fission yeast UCS protein (Rng3p) enhances actin binding by purified Myo2p. Here we tested Rng3p's role in promoting the function of newly synthesized Myo2p using the temperature-sensitive rng3-65 strain as an expression system. Loss of Rng3p function after initiation of Myo2p synthesis has little effect on its motor activity in motility assays. On the other hand, motors expressed in the absence of functional Rng3p can bind actin filaments but do not support filament gliding in these assays. A Myo2p motor mutant (Myo2-E1p) that is hyper-sensitive to defects in Rng3p function in vivo was studied. Myo2-E1p binds actin filaments but exhibits weak ATPase and motility rates. Thus, Rng3p apears to be important in establishing the activity of Myo2p motors. Myo2p levels were reduced in the rng3-65 background suggesting that Rng3p promotes Myo2p stability in the cell. We next tested regulation of Myo2p at the level of the actin filament by fission yeast tropomyosin (Cdc8p). Purified Cdc8p enhances Myo2p's affinity for actin filaments, but does not influence its overall rate of ATP hydrolysis. In actin-activated Myo2p ATPase assays, Cdc8p-decorated actin filaments reduce the KM, while having no effect on VMAX. No change in Myo2p's motility rate was apparent when using Cdc8p-decorated filaments. Collectively these findings suggest Rng3p and Cdc8p are both involved in ensuring effective myosin-II motor function during cytokinesis.

2703/B414

Novel Role for the Membrane Protein Gpibalpha in Cytokinesis and Tetraploidy in Cancer Cells.

F. Xu[1], Q. Wu[1], Y. Li[2], E. Prochownik[2,3], W. S. Saunders[1,2]; [1]Biological Sciences, University of Pittsburgh, Pittsburgh, PA, [2]Biochemistry and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA, [3]Oncology Research, Children's Hospital of Pittsburgh, Pittsburgh, PA

GpIbalpha is a subunit of von Willebrand factor receptor, functioning in promoting platelet adhesion and activation. Recent studies have revealed that GpIbalpha is also a classical oncoprotein, playing roles in transformation, genomic instability and senescence. In interphase cells, GpIbalpha was observed to colocalize with actin and filamin, an actin-binding protein. Moreover, cellular fractionation using HeLa cells demonstrated that GpIbalpha is a membrane-associated protein. When the actin network was disrupted by cytochalasin D, GpIbalpha no longer localized properly. Interestingly, in dividing cells we observed the colocalization of GpIbalpha and actin in the contractile rings. Cancer cells express high levels of GpIbalpha and when GpIbalpha was knocked down in HeLa, osteosarcoma and MCF-7 cancer cells, a decrease in multinucleation and mitotic defects was observed. Conversely, when we overexpressed GpIbalpha in an immortalized normal cell

line with p53 knockdown, about 10% of cytokinesis failure was observed as demonstrated by live cell imaging, showing that overexpression of GpIbalpha can lead to cytokinesis failure. Immunofluorescence analysis demonstrated that endogenous GpIbalpha does not localize to the contractile ring when GpIbalpha was overexpressed. Instead, abnormal morphologies were observed in dividing cells including pole contraction as well as blebbing; and actin, filamin and GpIbalpha mislocalized to these contractile blebs instead of the cleavage furrow. Taken together, our data suggest that GpIbalpha is a membrane protein associated with actin/filamin network and is involved in cytokinesis. Furthermore, overexpression of GpIbalpha is responsible for cytokinesis failure in cancer cells.

2704/B415

Laser Ablation of Telomeres in Anaphase Causes Delay in Cytokinesis.

N. Baker[1], S. Zeitlin[5, 4], L. Shi[2], J. V. Shah[6], M. Berns[3, 2]; [1]Electrical Engineering, University of California San Diego, San Diego, CA, [2]Bioengineering, University of California San Diego, San Diego, CA, [3]Bioengineering, University of California Irvine, Irvine, CA, [4]Cellular and Molecular Medicine, University of California San Diego, San Diego, CA, [5]Ludwig Institute, University of California San Diego, San Diego, CA, [6]Harvard Medical School, Harvard, Boston, MA

During cell division, damaged DNA can result in mitotic arrest, which can lead to cell death. How DNA damage communicates with mitotic progression machinery, however, remains poorly understood. Through the use of laser-mediated ablation, we have targeted chromosomal regions for ablation at various times in mitosis. Here we demonstrate that the ablation of chromosome tips, presumptive telomeric domains, during anaphase in *Potorous tridactylus* cells (PTK2), using a picosecond laser (532 nm, 76 Mhz, 12 picosecond, Irradiance 0.49 x 10^{10} W/cm^2, over 0.17 um^2), resulted in defects in cytokinesis. Specifically we observed three phenotypes: (1) Prolonged delay in furrow formation (hour), followed by a normal cytokinesis (2) Furrow ingression, regression and a 2nd ingression after few hours, followed by successful cytokinesis. (3) No furrow ingression observed followed by cell death. Moreover, ablation of non-telomeric regions in anaphase caused no significant delay in the timing and completion of cytokinesis. These results raise the possibility of a checkpoint arrest mechanism that links dysfunctional telomeres to inhibition of the cytokinetic furrow. This may be linked to a known DNA damage pathway, or an unknown telomere specific surveillance pathway.

2705/B416

Aneuploidy Driving Rapid Phenotypic Evolution of an Essential Cell Division Process.

N. Pavelka, G. I. Rancati, B. Fleharty, A. Noll, R. Allen, K. Walton, A. Perera, K. Staehling-Hampton, C. Seidel, R. Li; Stowers Institute for Medical Research, Kansas City, MO

A growing body of evidence suggests that processes such as tumorigenesis and development of drug resistances are outcomes of cellular evolution. To better understand cellular evolvability, defined as the capacity of cells to adjust to perturbations through evolutionary processes, we used a unique experimental design to assess the evolutionary response of yeast cells to the deletion of an essential gene involved in cytokinesis. Yeast cells deleted of the MYO1 gene, encoding the only type II myosin in the yeast genome, initially exhibited severe growth and cytokinesis defects but were able to rapidly evolve alternative pathways to restore both processes. Detailed cell biological analyses revealed the emergence of several different mechanisms to achieve closure of the mother-bud junction. Transcriptome profiling indicated that the evolved cytokinesis mechanisms correlated with and relied upon gene expression changes in specific pathways with no obvious direct relationship to Myo1-dependent functions. To identify the genetic changes responsible for the gene expression changes in the myo1Δ e-strains, we used a series of genome analysis methods, including comparative genomic hybridization and whole-genome re-sequencing. The results revealed a strong correlation between favorable evolutionary outcome and polyploidization and aneuploidization, which are genomic aberrations often observed in cancer cells. Aneuploidy could account for not only gene expression changes quantitatively correlated with chromosome stoichiometries but also changes far beyond this effect. We finally show how changes in gene copy numbers of specific regulatory molecules, which mimic the effect of aneuploidy, can recapitulate one of the evolved cytokinesis phenotypes in myo1Δ cells. Taken together, these results demonstrate how cells can rapidly evolve to seemingly irreversible perturbations by tinkering with gene copy number stoichiometry caused by whole-chromosome aneuploidy.

Kinetochores II (2706 – 2727)

2706/B417

Spindle Pole Accumulation of Mitotic Checkpoint Proteins Maintains Mitotic Checkpoint Silencing.

J. Famulski[1], J. B. Rattner[3], G. Chan[1,2]; [1]Oncology, University of Alberta, Edmonton, AB, Canada, [2]Experimental Oncology, Cross Cancer Institute, Edmonton, AB, Canada, [3]Anatomy and Cell Biology, University of Calgary, Calgary, AB, Canada

The mitotic checkpoint functions to ensure accurate chromosome segregation by regulating the progression from metaphase to anaphase (Chan et al, 2005; Musacchio & Salmon, 2007). Once the checkpoint has been satisfied, it is inactivated in order to allow the cell to proceed into anaphase and complete the cell cycle. The minus end-directed microtubule motor dynein/dynactin has been implicated in the silencing of the mitotic checkpoint by "stripping" checkpoint proteins off kinetochores (Howell et al, 2001; Wojcik et al, 2001). Here we examine the effects of the small molecule Nordihydroguaiaretic acid (NDGA), on the dynein/dynactin dependent transport of mitotic checkpoint proteins from kinetochores to spindle poles, and the status of the mitotic checkpoint. hZW10 is a known cargo of dynein/dynactin and was therefore used as a readout of its transport activity. We observed the transport of EGFP-hZW10 in live cells treated with NDGA and determined that it requires kinetochore-microtubule (k-MT) attachments but not tension, bi-polar attachments or Aurora B kinase activity. hZW10 is a dynamic component of the spindle pole during mitosis, however, hZW10 accumulates at spindle poles and continues to accumulate in the presence of NDGA. We also found that the transport and accumulation of hROD, hSpindly, hMad1, hMad2, hBubR1, hCENP-E, hCdc27, Cyclin-B and hMps1 at spindle poles in cells treated with NDGA. Surprisingly, NDGA treatment did not silence the mitotic checkpoint but instead induced a mitotic checkpoint dependent arrest without affecting kinetochore-MT attachment or tension. We therefore propose, that in addition to

dynein/dynactin mediated transport of checkpoint proteins off kinetochores, the dissociation of mitotic checkpoint proteins off spindle poles is required for silencing of the mitotic checkpoint.

2707/B418

MPS1 and PLK1 Have Overlapping Substrate Specificities, but Only MPS1 Can Regulate Cenp-E Motility.

A. Gaussen[1], J. Espeut[1], S. Prieto[1], N. Sommerer[2], V. Morin[1], D. Fesquet[1], S. Merlot[3], M. Rossignol[2], A. V. Kajava[1], A. Abrieu[1]; [1]CRBM, CNRS, Montpellier, France, [2]LPF, INRA, Montpellier, France, [3]ISV, CNRS, Gif-sur-Yvette, France

MPS1 kinase is essential for processes driven by the kinetochore, such as chromosome alignment and activation of the checkpoint that ensures accurate sister chromatid separation, but few substrates are known. Here, we define MPS1 target preference using a semi-degenerate peptide array and rationally designed peptide substrates. We also analyzed MPS1 phosphorylation of Cenp-E, a kinetochore motor that we have previously shown to be regulated by MPS1 via direct phosphorylation of its tail domain. We identified multiple MPS1 phosphorylation sites on Cenp-E and characterized the sites required to regulate Cenp-E motility using microtubule-gliding assays. An analysis of these data as well as data from all other known MPS1 substrates leads to the conclusion that MPS1 is an acidophilic, proline-aversive kinase. The main feature of the MPS1 phosphorylation consensus is a preference for acidic amino acids N-terminal of the phosphorylated site, as with PLK1, and a preference for threonine rather than serine as phosphate acceptor. We show that although MPS1 and PLK1 can phosphorylate some common sites on Cenp-E, only MPS1 relieves autoinhibition of Cenp-E motility. This reflects our finding that MPS1 is more promiscuous than PLK1, a characteristic that we rationalize by molecular modeling of MPS1 and PLK1 kinase domains.

2708/B419

Sensing Centromere Tension by Spatial Separation of Aurora-B Kinase from Kinetochore Substrates.

D. Liu[1], G. Vader[2], M. J. M.Vromans[2], S. M. A. Lens[2], M. Lampson[1]; [1]Department of Biology, University of Pennsylvania, Philadelphia, PA, [2]Department of Medical Oncology, University Medical Center Utrecht, Universiteitsweg, Netherlands

Aurora-B is an inner centromere kinase which is responsible for regulating attachments between kinetochores and spindle microtubules to ensure accurate chromosome segregation. Phosphorylation of Aurora B substrates at the kinetochore, including Dam1 and the NDC80 complex, decreases affinity for microtubules. Aurora B selectively destabilizes incorrect attachments while correct attachments, which are under tension, remain stable. How Aurora B distinguishes correct and incorrect attachments is an important unanswered question. We considered two models to explain how selective destabilization is achieved. In the first model tension inactivates Aurora-B on bi-oriented centromeres. In the second model tension separates kinetochore substrates from Aurora B at the inner centromere, which reduces phosphorylation on bi-oriented kinetochores. To distinguish between these models, we used FRET-based sensors that report on phoshorylation of an Aurora B substrate in living cells. Sensors were targeted to centromeres or kinetochores to measure localized phosphorylation changes. Cells were treated with nocodazole, monastrol or MG132 to measure steady-state phosphorylation in different attachment states. We also measured phosphorylation dynamics during the correction of attachment errors. The centromere-targeted sensor is constitutively phosphorylated, independent of microtubule attachments, while the kinetochore-targeted sensor is phosphorylated only in the absence of tension. These results suggest that phosphorylation of an Aurora B substrate depends on its distance from the kinase. We also constructed INCENP fusion proteins to reposition Aurora B from the inner centromere closer to the kinetochore, which prevents stabilization of bi-oriented attachments and activates the spindle checkpoint. Our results provide direct evidence for a mechanism to sense centromere tension through spatial separation of Aurora B from kinetochore substrates, which reduces substrates phosphorylation and stabilizes kinetochore microtubules attachments.

2709/B420

Role of Hec1 N-Terminus in Kinetochore-Microtubule Attachment and Outer Plate Ultrastructure.

Y. Dong[1], G. Guimaraes[2], X. Meng[1], J. DeLuca[2], B. F. McEwen[1]; [1]Division of Molecular Medicine, Wadsworth Center, NYS Dept Health, Albany, NY, [2]Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO

Proper segregation of sister chromatids during mitosis depends on the formation of kinetochore-microtubule (MT) attachments. The kinetochore is a proteineous complex composed of three domains: an inner domain, a stable outer domain and a dynamic outer domain. Electron microscopy has established that the stable outer domain, also known as the outer plate, is a 50 nm thick disc that contains a flexible fibrous network. The outer plate is the major attachment and termination site for kinetochore MT (kMT) plus ends. The Hec1 complex, a heterotetramer located in the outer plate, functions both in forming kMT attachments and maintaining the structural integrity of the outer plate network. Here we used transmission electron microscopy (TEM) to investigate the role of the N-terminus of Hec1 protein in forming kinetochore-MT interactions and sustaining kinetochore ultrastructure. As previously observed in HeLa cells, Hec1 depletion using RNAi nearly abolished kMT attachments in PtK1 cells. PtK1 cells were used because they are well-suited for correlative light microscopy (LM) and TEM. KMT attachment was rescued in PtK1 cells transfected with full length Hec1, but not in cells transfected with mutant Hec1 protein lacking the N-terminal 80 amino acids. Transfection with an N-terminal nonphosphorylatable Hec1 mutant resulted in robust kMT attachment. This observation is consistent with previous work suggesting that Aurora B phosphorylation destabilizes inappropriate kMT attachments that lead to erroneous chromatid segregation. In contrast to HeLa cells, however, the outer plate structure is detectable in Hec1 depleted PtK1 cells. We are currently using electron tomography to determine how the Hec1 subdomains affect the outer plate ultrastructure and its function in PtK1 and HeLa cells.

2710/B421

Bir1 Is Required for the Tension Checkpoint.

P. O. Widlund[1,3], M. M. Shimogawa[1,2], M. Riffle[1], M. Ess[1], T. Davis[1,2]; [1]Biochemistry, University of Washington, Seattle, WA, [2]Program in Molecular & Cellular Biology, University of Washington, Seattle, WA, [3]Max Planck Institute of Molecular Cell Biology & Genetics, Dresden, Germany

The *Saccharomyces cerevisiae* chromosomal passenger proteins Ipl1 (Aurora B) and Sli15 (INCENP) are required for the tension checkpoint, but the role of the third passenger, Bir1, is controversial. We have isolated a temperature-sensitive mutation (*bir1-107*) in the essential C-terminal region of Bir1 known to be required for binding to Sli15. This allele reveals a checkpoint function for Bir1. The mutant displays a biorientation defect, no cell cycle delay in response to lack of tension, and an inability to detach mutant kinetochores. Ipl1 localizes to aberrant foci when Bir1 localization is disrupted in the *bir1-107* mutant. Thus, one checkpoint role of Bir1 is to properly localize Ipl1 and allow detachment of kinetochores. Quantitative analysis indicates that the chromosomal passengers colocalize with kinetochores in G1, but localize between kinetochores that are under tension. Bir1 localization to kinetochores is maintained in an *mcd1-1* mutant in the absence of tension. Our results suggest that the establishment of tension removes Ipl1, Bir1 and Sli15, and their kinetochore detachment activity, from the vicinity of kinetochores and allows cells to proceed through the tension checkpoint.

2711/B422

The Spatio-Temporal Composition of the Chromosomal Passenger Complex (CPC) in Mitosis Analysed Using In Situ Proximity Ligation Technique.

M. Vuoriluoto[1], L. Ahonen[1], J. Pouwels[1], R. Mäkelä[2], P. Saviranta[2], M. Kallio[1,2,3]; [1]Turku Centre for Biotechnology, Turku, Finland, [2]VTT Medical Biotechnology, Turku, Finland, [3]Centre of Excellence for Translational Genome-Scale Biology, Academy of Finland, Finland

Proximity ligation assay (PLA) is a novel method enabling study of protein-protein associations with spatio-temporal resolution. The assay is based on detection of primary protein antibodies with proximity probes composed of secondary antibody and a connector oligonucleotide. The probes bind with the primary antibodies attached to target proteins of interest. In case the two proteins are in close proximity the connector oligonucleotides of the probes ligate and form a circular DNA molecule that is amplified by rolling-circle amplification (RCA). Lastly, the RCA product is fluorescently labelled. The Chromosomal Passenger Complex (CPC) comprising the catalytic subunit Aurora-B kinase and three non-enzymatic subunits (Incenp, Survivin and Borealin) is essential for many mitotic functions ranging from promotion of correct microtubule-kinetochore attachments to execution of cytokinesis. We have utilized the PLA to study the composition of the CPC in different phases of cell division. The CPC probe pairs show a dotted labelling pattern at centromeres in early mitosis and at the spindle midzone during anaphase and telophase, which corresponds to the known subcellular localizations of CPC proteins. Extensive analysis of PLA signals in five mitotic phases with various CPC protein pairs such as Aurora B-Survivin, Aurora B-Incenp, Aurora B-Borealin, and Survivin-Borealin proposes that the core complex is intact and present in all phases of mitosis. Depletion of Incenp by siRNA abolished the PLA signals of all CPC protein pairs indicating that PLA signals are specific and not formed when the complex is mislocalized from the centromeres. Removal or hyperstabilization of microtubules did not affect the amount of PLA signals indicating that the complex composition was not altered by the absence of proper microtubule-kinetochore attachments or inter-kinetochore tension. In conclusion, we have shown that PLA can be used to study protein-protein interactions at specific mitotic organelles and CPC forms a tight complex in early mitosis that persists throughout the M-phase. We are currently exploring the possibility to use PLA as readout in high-throughput siRNA screens.

2712/B423

Condensin Is Essential for Centromeric Chromatin Assembly and Sister Kinetochore Orientation in Anaphase.

A. Samoshkin[1], A. Arnaoutov[1], L. Jansen[3], I. Ouispenski[1], L. Dye[1], T. Karpova[2], J. McNally[2], M. Dasso[1], D. Cleveland[3], A. Strunnikov[1]; [1]NIH, NICHD, Bethesda, MD, [2]NIH, NCI, Bethesda, MD, [3]Ludwig Institute For Cancer Research, UCSD, La Jolla, CA

The establishment and maintenance of chromosome condensation are essential cell cycle steps, which facilitate accurate chromosome segregation and therefore ensure the integrity of eukaryotic genomes. Condensins I and II in vertebrates are essential ATP-dependent enzyme complexes necessary for chromosome condensation in mitosis. Depletion of condensin activity is now shown to result in a significant loss of loading of CENPA, the histone H3 variant found at active centromeres and the proposed epigenetic mark of centromere identity. Paradoxically, loss of condensin also activated the mitotic checkpoint (also known as the spindle assembly checkpoint). Stretching of centromeric chromatin by spindle forces after condensin depletion produced deformation of inner kinetochores and loss of the HEC1/Ndc80 microtubule-capturing module, as well as aurora B mislocalization and reduced kinase activity. Recovery from monastrol revealed a high incidence of merotelic attachment that was nearly identical to (and epistatic with) cases of condensin depletion and Aurora B inactivation. Thus, beyond a requirement for global chromosome condensation, condensin plays a pivotal role in kinetochore assembly including proper spatial positioning of microtubule capture and positioning complexes of the inner and outer centromeres, respectively.

2713/B424

Condensin Regulates the Stiffness of the Vertebrate Centromere.

S. A. Ribeiro[1], J. Gatlin[2], Y. Dong[3], A. Joglekar[2], L. Cameron[2], B. McEwen[3], T. Salmon[2], W. Earnshaw[1], P. Vagnarelli[1]; [1]Wellcome Trust Centre for Cell Biology, Edinburgh, United Kingdom, [2]Dep of Biology, University of North Carolina, Chapel Hill, NC, [3]Wadsworth Center, New York State Department of Health, Albany, NY

Chromosome alignment in metaphase is the result of an equilibrium of forces generated by the mitotic spindle, the chromosomes and the molecular effectors that mediate the connection between kinetochores and microtubules. Kinetochores remain attached to microtubule tips even during assembly and disassembly. This generates the back-and-forth movements of the sister kinetochores on the metaphase plate known as oscillations. Tension opposes the pulling forces of the spindle. When all the chromosomes are oriented and under tension, the spindle checkpoint is inactivated and anaphase occurs. Using a conditional knock-out for the condensin subunit SMC2 in DT40 cells we have performed detailed live cell imaging analyses of kinetochore behavior in metaphase cells in the presence and absence of condensin (I and II). In colcemid-blocked cells without condensin, the distance between sister kinetochores is undistinguishable from the controls, revealing that condensin is dispensable for the condensation (rest length) of centromeric heterochromatin during mitosis. However, the kinetochores of chromosomes aligned on the metaphase plate exhibited an abnormal mobility. Single kinetochores could be seen as far as 2-3 µm away from the chromosomes, to which they were linked by a thin chromatin thread. During these excursions, only the inner centromeric hetero-chromatin but not the kinetochores were subjected to deformations, which were reversible. From this study we propose a model where the

rest length of the centromere is established by the higher order chromatin structure while the stiffness (expressed as spring constant) is regulated by condensin. Therefore condensin represents the compliant element for the vertebrate centromere and it is necessary for the balance of the pulling forces generated by spindle microtubules in metaphase.

2714/B425

Characterizing the Kinetochore Function of *S. Cerevisiae* CLASP

M. Hou; Systems Biology, Harvard Medical School, Boston, MA

Chromosome segregation at mitosis is crucial for the maintenance of genomic integrity in eukaryotic cells. To ensure high-fidelity segregation, the accurate assembly of kinetochores and their stable attachment to microtubules are necessary. Saccharomyces cerevisiae kinetochores are complex proteinaceous structures containing 60 protein components assembled at the centromeric DNA nucleosomes containing the histone H3 variant Cse4p through a scaffold protein complex CBF3 (Ndc10p, Cep3p, Ctf13p and Skp1p). In addition to those core kinetochore components, some microtubule associated proteins (MAPs) also localize to kinetochores. The MAPs have an important function in regulating spindle assembly and dynamics, but the understanding of their role at the kinetochore is limited. To understand whether the kinetochore-bound MAPs (kMAPs) play a crucial role in kinetochore-microtubule interaction, we investigate the function of the kMAP Stu1p. Stu1p is a member of a conserved protein family (CLASP). It is required for spindle pole body separation and spindle assembly. Our study and others have shown that Stu1p localizes to kinetochores. We find that Stu1p accumulates specifically at unattached kinetochores in response to nocodazole, which is co-localized with the unattached kinetochore-bound Ndc80p and the checkpoint proteins Mad2p and Bub1p. Furthermore, we find that the localization of the kMAPs examined in response to nocodazole fall into three classes: (i) close to the collapsed the SPBs (Bim1p, Bik1p and Cin8p) (ii) close to the SPBs and unattached kinetochores (Ndc80p, Dam1p and Stu2p) (iii) unattached kinetochores (Stu1p). The results suggest that the unattached kinetochore localization is a unique characteristic to Stu1p. The unattached kinetochore localization of Stu1p does not depend upon Mad2p and Bub1p, suggesting that the activation of the spindle checkpoint is not required for recruiting Stu1p to the unattached kinetochores. Thus, Stu1p may function as a marker for unattached kinetochores, required for re-attachment of kinetochores to microtubules, a hypothesis that is currently under investigation.

2715/B426

Identification and Characterization of New Kinetochore Proteins.

A. A. Tavares; [1]Cell Division Group, Instituto Gulbenkian Ciencia, Oeiras, Portugal, [2]Dept Chemical Biological Eng, Inst. Superior Tecnico, Lisboa, Portugal

We have developed a new method to obtain enriched mitotic kinetochore preparations from Drosophila embryos and cultured cells. From such preparations we have identified new kinetochore components by analysis on 2D-gels and by peptide mass fingerprinting. This way we have identified 92 proteins of which 26 correspond to previously uncharacterized genes. We have named these genes Kpd1 to Kpd26 (Kinetochore Preparation from Drosophila). Expression of these proteins as GFP-fusions in Drosophila S2 cultured cells, showed that 10 do accumulate of the mitotic kinetochores. In addition, downregulation by RNAi of the Kpd proteins results in severe chromosome congression and segregation defects. In agreement with these results, preliminary mutant analysis for four of new the Kpd genes show that they are essential in early embryonic or larval development. Finally, we have identified the human homologues for Kpd1, Kpd2 and Kpd12 (undescribed in humans), and downregulation of these genes in HeLa cells also resulted in severely defective chromosome congression and segregation during mitosis. Taken together, our results indicate that we have identified a new set of proteins required for mitotic kinetochore function in Drosophila and humans cells. In addition, the results also re-inforce the idea that the components of Drosophila kinetochore are conserved across species.

2716/B427

Kinetochore-Generated Pushing Forces Can Separate Centrosomes during Bipolar Spindle Assembly.

A. Toso[2], J. Winter[1], A. Amaro[1], P. Meraldi[2], A. McAinsh[1]; [1]MCRI, The Chart Oxted, United Kingdom, [2]ETH, Zürich, Switzerland

In animal somatic cells bipolar spindle formation requires the separation of the centrosome-based spindle poles. Centrosomes can either migrate in opposite directions during prophase by interacting with the nuclear membrane or they can be separated after nuclear envelope breakdown during prometaphase by microtubule sliding and by forces produced by cortical attachments. We have previously shown that depletion of the human kinetochore protein Mcm21R (also known as CENP-O) causes monopolar spindles, suggesting a role for kinetochores in bipolar spindle formation. Here we show that Mcm21R, and kinetochores in general, are required for rapid centrosome separation in subset of cells that do not separate their centrosomes before nuclear envelope breakdown. Our data indicate that kinetochores push the centrosomes apart during prometaphase by exerting a reactive force on the k-fibers, the bundles of microtubules that connect kinetochores to centrosomes. This pushing force is based on microtubule poleward flux and requires kinetochores to stabilize k-fibers in order to transmit the force. Interestingly, this k-fiber-dependent pushing force becomes essential for centrosome separation and bipolar spindle assembly if the Aurora-A protein kinase, a crucial regulator of centrosomes, is inhibited. We conclude that two distinct mechanisms exist for pole-separation during prometaphase, an Aurora-A-dependent pathway and an Aurora-A-independent, kinetochore-dependent pathway that relies on k-fiber-generated pushing forces.

2717/B428

Reconstitution of Microtubule-Driven Movement and Force Production by the Ndc80 Kinetochore Complex.

A. Powers[1], A. D. Franck[1], D. R. Gestaut[2], J. Cooper[1], B. Gracyzk[2], R. Wei[3,4], L. Wordeman[1], T. Davis[2], C. Asbury[1]; [1]Physiology and Biophysics, University of Washington, Seattle, WA, [2]Biochemistry, University of Washington, Seattle, WA, [3]Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA, [4]Genzyme Corporation, Framingham, MA

Kinetochores maintain load-bearing attachments between chromosomes and microtubule tips, even as the tips assemble and disassemble under their grip. This end-on coupling is fundamental to the many other roles kinetochores perform during mitosis, such as controlling the growth and shortening of attached microtubules, sensing and correcting errors in attachment, and emitting a diffusible 'wait' signal until they

are properly attached. End-on coupling is also conserved from yeast to humans, suggesting it may be mediated by a common molecular mechanism. However, the existence of a conserved coupling mechanism remains uncertain. Using techniques for manipulating and tracking individual molecules, we discovered that end-on coupling can be reconstituted with the Ndc80 complex, a widely conserved component of the outer kinetochore. Beads decorated with pure Ndc80 complex bind individual microtubules through the N-terminal globular domains of Ndc80p and Nuf2p. Like kinetochores, these Ndc80-based couplers are carried by disassembling tips and they remain tip-bound even under tensile loads up to 2.5 pN. Microtubule-driven movement and force production persist at low surface densities where the number of Ndc80 complexes interacting with the filament is close to the number found at kinetochores in vivo. At the single molecule level, fluorescent-tagged Ndc80 complexes diffuse randomly along microtubules except at disassembling tips where their motion becomes biased in the direction of filament shortening. Our results indicate that Ndc80-based coupling likely depends upon a 'fluctuating ensemble' mechanism similar to that proposed by Hill on purely theoretical grounds more than two decades ago*. We also demonstrate that both the yeast and human Ndc80 complexes support microtubule-driven bead movement and force production. This conservation of function, together with the established importance of the Ndc80 complex for kinetochore-microtubule coupling across eukaryotes, strongly suggests that a fluctuating ensemble of Ndc80 complexes represents a core attachment module within the kinetochore. * Hill, T. L. (1985) Proc Natl Acad Sci USA 82:4404-4408.

2718/B429

CENP-E Combines a Slow, Processive Motor and a Flexible Coiled-Coil to Produce an Essential, Motile Kinetochore Tether.

Y. Kim[1], J. Heuser[2], C. M. Waterman[3], D. Cleveland[1]; [1]Ludwig Institute for Cancer Research, University of California, San Diego, La Jolla, CA, [2]Department of Cell Biology, Washington University, St Louis, MO, [3]The National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD

The mitotic kinesin CENP-E is an essential kinetochore component that directly contributes to the capture and stabilization of spindle microtubules by kinetochores. Although reduction in CENP-E leads to high rates of whole chromosome missegregation, neither its properties as a microtubule-dependent motor nor how it contributes to the dynamic linkage between kinetochores and microtubules is known. Using single molecule assays, we demonstrate here that CENP-E is a very slow, highly processive motor that maintains microtubule attachment for long periods. Direct visualization of full-length Xenopus CENP-E reveals a highly flexible, 230 nm coiled-coil separating its kinetochore binding and motor domains. We also show that full-length CENP-E is a slow plus end-directed motor whose activity is essential for metaphase chromosome alignment. We propose that the highly processive microtubule-dependent motor activity of CENP-E serves to power chromosome congression, and provides a flexible, motile tether linking kinetochores to dynamic spindle microtubules.

2719/B430

hZwint-1 and hZw10 Localization and Expression Are Altered in a Subset of Breast Cancer Cell Lines.

L. Vos[1], G. Chan[2,1]; [1]Oncology, University of Alberta, Edmonton, AB, Canada, [2]Experimental Oncology, Cross Cancer Institute, Edmonton, AB, Canada

Cancer is characterized by uncontrolled cell proliferation and many solid tumours exhibit aneuploidy. The mechanism(s) that leads to these phenotypes is not fully understood. Because the mitotic checkpoint ensures accurate segregation, it has been proposed that loss or weakening of the mitotic checkpoint could produce aneuploidy. Both hZwint-1 and hZw10 are required for accurate chromosome segregation as cells depleted of hZwint-1 or hZw10 have misaligned chromosomes, premature anaphase and checkpoint escape after MT poison treatment resulting in multinucleated cells. hZwint-1 is overexpressed in a subset of pulmonary adenocarcinomas with poor prognosis (Endoh et al. (2004) J Clin Oncol). To determine whether this was a more general trend in cancer we examined hZwint-1 protein levels from non-tumorigenic and cancer cell lines by Western blotting. From the cell lines examined hZwint-1 levels are higher in cancer cell lines. Subcellular localization analysis by immunofluorescence has shown there are also differences in hZwint-1 kinetochore localization timing. In MCF10A, a non-tumorigenic breast cell line, hZwint-1 localizes to the kinetochore from G2 through mitosis until cytokinesis while in the breast cancer cell lines MCF7 and MDA-MB-468, hZwint-1 does not localize to the kinetochore until prophase and leaves during anaphase B. While examining the localization of hZwint-1 in breast cancer cell lines we also looked at hZw10 localization and found that compared to HeLa and MCF10A cells, hZw10 is absent from or only weakly present on prometaphase kinetochores of several breast cancer cell lines. When cells were treated with vinblastine, hZw10 accumulated at the kinetochore, indicating that while hZw10 is capable of localizing the regulation of kinetochore occupancy is abnormal. We are examining whether hZw10 is mutated in these breast cancer cell lines exhibiting reduced kinetochore occupancy as well as determining if the interactions between hZw10 and its binding partners hRod, hZwilch and hZwint-1 are affected. The majority of the breast cancer cell lines examined are aneuploid and we are examining whether differences in hZwint-1 and hZw10 expression and localization results in a weakened mitotic checkpoint.

2720/B431

Understanding the Mitotic Role of MEI-S332 Protein in Drosophila Melanogaster.

A. F. Sousa, P. A. Coelho, C. E. Sunkel; Molecular Genetics, Instituto de Biologia Molecular e Celular, Porto, Portugal

During cell division accurate distribution of the genome requires that sister chromatids remain cohered with sister kinetochores bipolarly attached to the spindle at metaphase-anaphase transition. This correct attachment generates tension on centromere, inactivating Spindle Assembly Checkpoint pathway allowing ultimately the cleavage of SCC1/RAD21 cohesin subunit by separase which triggers anaphase onset and segregation of the sister chromatids to opposite poles. The Drosophila MEI-S332 protein is the founding member of Shugoshin family of proteins and has been originally described as a centromeric cohesion protector during meiosis I. This is a conserved role already proven to be played by members of this family from yeasts to mammals. Our goal is understand MEI-S332 role in mitosis where it localises at the centromeric region since prophase till metaphase-anaphase transition. This localisation is dependent of checkpoint proteins as in BubR1-depleted cells Mei-S332 is no longer observed at the primary constriction but dispersed over the chromatin. In vivo time-lapse microscopy of Drosophila cells stably expressing mcherry-CID and GFP-tubulin indicates that after dsRNAi for MEI-S332 chromosomes are able to align to metaphase plate with no separation of sister chromatids although a delay is measured at this stage. Eventually, cells are able to progress into anaphase without segregation defects. Although several shugoshin members are reported to harbour protective role of the cohesion

complex at the centromere, SCC1/RAD21 localises at the inner centromere region at normal levels in the absence of MEI-S332. This observation indicates that MEI-S332 is not required to avoid RAD21/SCC1 cleavage during mitosis. It was also demonstrated that another paralogue of human Shugoshin, Sgo2, is important for chromosome biorientation and controls docking of the Passenger proteins on chromosomes in early mitosis, our preliminary results show that INCENP is absent in MEI-S332 depleted cells indicating that in this aspect MEI-S332 is closer to Sgo2 than to SgoI.

2721/B432

Kinesin-Mediated Control of Mitotic Chromosome Congression.

J. Stumpff[1], G. Von Dassow[2], C. Huseby[1], C. Asbury[1,2], L. Wordeman[1,2]; [1]Physiology and Biophysics, University of Washington, Seattle, WA, [2]Center for Cell Dynamics, University of Washington, Friday Harbor, WA

During mitosis in vertebrate cells, chromosomes oscillate with periods of smooth motion interrupted by abrupt reversals in direction. The molecular mechanisms that spatially constrain these movements to facilitate the alignment and segregation of chromosomes with high fidelity are not well understood. Our studies indicate that two kinesin-like motors, Kif18A and Kid, play complimentary roles in regulating mitotic chromosome positioning in human cells. Depletion of either Kif18A or Kid results in abnormal chromosome alignment and their co-depletion further increases the severity of positioning defects. Quantitative analyses of kinetochore movements reveal that Kif18A and Kid differentially affect pre-anaphase oscillations. Depletion of Kif18A increases oscillation amplitude and decreases the rate of directional switches while depletion of Kid reduces oscillation amplitude and increases the rate of directional reversals. In cells depleted of both kinesins, kinetochores oscillate with reduced amplitude compared to Kif18A-depleted cells despite having more severe alignment defects. These results suggest that two kinesin-dependent mechanisms may act in parallel to regulate chromosome movement and position them at the spindle equator.

2722/B433

Kinetochore-Microtubule Attachment Relies on the Disordered N-Terminal Tail Domain of Hec1.

G. Guimaraes[1], Y. Dong[2], B. McEwen[2], J. DeLuca[1]; [1]Biochemistry and Molecular Biology, Colorado State University, Fort Collins, CO, [2]Wadsworth Center, New York State Department of Health, Albany, NY

Accurate chromosome segregation is dependent upon kinetochores stably attaching chromosomes to spindle microtubules during mitosis. A critical and long-standing question is how kinetochores are able to maintain stable attachment to the plus-ends of dynamic microtubules that are continually growing and shrinking. The hetero-tetrameric Ndc80 complex (Ndc80/Hec1, Nuf2, Spc24, and Spc25) is essential for persistent, end-on kinetochore-microtubule attachment in cells, and recent studies have shown that it is able to bind directly to microtubules in vitro. How the Ndc80 complex attaches to microtubules to form functional binding sites in cells remains unknown. Here we use a silence and rescue strategy, in which PtK1 cells are co-transfected with Hec1 siRNA and Hec1-GFP expression constructs, to demonstrate that the N-terminus of Hec1 is essential for generating stable kinetochore-microtubule attachments. PtK1 cells expressing Hec1-GFP fusion proteins deleted of the entire N-terminal globular domain or of the disordered N-terminal 80 amino acid "tail" domain fail to bi-orient their chromosomes, establish tension across sister kinetochore pairs, or generate end-associated kinetochore-microtubule attachments. Additionally, we demonstrate that mutation of 9 amino acid residues within the 80 amino acid tail domain to reduce its positive charge abolishes stable kinetochore-microtubule attachment. The mitotic checkpoint remains functional after deletion of the N-terminal 80 amino acids, but not the N-terminal 207 amino acids. These results demonstrate kinetochore-microtubule binding is dependent on electrostatic interactions mediated through the disordered N-terminal 80 amino acid tail domain and mitotic checkpoint function is dependent on the CH domain of Hec1.

2723/B434

An Unstructured Tail of Hec1/Ndc80 Directly Connects the Kinetochore to Microtubules.

S. Miller, P. Stukenberg; Biochemistry and Molecular Genetics, University of Virginia, Charlottesville, VA

Kinetochores bind and regulate the plus-ends of microtubules during mitosis. These attachments must be tight enough to move chromosomes, while the microtubule end both remains dynamic and repositions within the attachment pocket so that connections are maintained during depolymerization. Hec1/Ndc80 (Hec1) is a subunit of the four-protein Ndc80 complex, which is an important structural component of the kinetochore . Kinetochores are unable to bind microtubules after Hec1 knockdown ; however, because the Ndc80 complex has structural roles, it is unclear if Hec1 directly mediates kinetochore microtubule attachments. Hec1 has a microtubule-binding site composed of both an unstructured N-terminal tail and a calponin homology domain . Here we show that, surprisingly, the N-terminal tail is sufficient for microtubule binding affinity in vitro. The interaction is salt sensitive and the positively charged Hec1 tail is unable to bind microtubules lacking negatively charged tails. We have replaced the endogenous Hec1 subunit with a mutant lacking the N-terminal tail. These cells assemble kinetochores properly but are unable to congress chromosomes, generate tension across sister kinetochores, or establish cold-stable kinetochore-microtubule attachments. Our data argue that coulumbic attractions between two unstructured domains underlie the key interaction between the kinetochore and microtubules. We discuss the importance of this finding for models of repositioning of microtubules in the kinetochore during depolymerization.

2724/B435

A Dam1-Based Minimal Kinetochore Is Sufficient to Promote Chromosome Segregation in Budding Yeast.

E. Kiermaier[1], S. Woehrer[1], C. Peng[2], S. Westermann[1]; [1]Research Institute of Molecular Pathology, Vienna, Austria, [2]Molecular and Cell Biology, UC Berkeley, Berkeley, CA

Kinetochores are large multi-protein complexes that mediate chromosome segregation in all eukaryotes by dynamically connecting specialized chromosome regions, termed centromeres, to the plus-ends of spindle microtubules. Even the relatively simple kinetochores of the budding yeast Saccharomyces cerevisiae consist of more than 80 proteins, making analysis of their respective roles a daunting task. Here, we have developed a system that allows us to artificially recruit proteins to DNA sequences and ask whether they can provide kinetochore

function. Using this system we show that artificial recruitment of the microtubule-binding Dam1 complex to the CDEII element of yeast centromere DNA is sufficient to fully restore the mitotic stability of functionally compromised centromeres. The CDEII-Dam1 artificial kinetochore effectively competes with a wild-type kinetochore on a di-centric mini-chromosome while its function depends on an intact CDEIII element. Strikingly, artificial recruitment of Dam1, but not of other microtubule-binding proteins, to a plasmid lacking any centromere DNA is sufficient to confer its mitotic stabilization. We have used live cell microscopy to directly visualize mini-chromosome segregation mediated by a Dam1-based minimal kinetochore. Thus, it is possible to build a simple eukaryotic kinetochore by directly recruiting a microtubule force-transducing component to DNA.

2725/B436

Molecular Delineation of Ndc80 Complex Assembly Dynamics and Plasticity in Mitosis.

X. Cai[1,2], Z. Wang[1], K. Yuan[2,1], S. Hua[1], Y. Huang[1], N. Li[1], K. Johnsson[3], X. Yao[1]; [1]Cell Biology, Hefei National Laboratory, Hefei, China, [2]Physiology, Morehouse School of Medicine, Atlanta, GA, [3]Chemical Sciences and Engineering, Ecole Polytechnique Fédérale de Lausanne, Lausanne, Switzerland

Mitosis is a highly dynamic process in which the chromosomes are segregated by orchestrated interaction of kinetochores with spindle microtubules. Kinetochore is a protein superstructure assembled onto centromere of the chromosome. We and other recently identified regulatory pathways that govern Ndc80 complex plasticity essential for faithful kinetochore-microtubule interaction in mitosis. These studies illustrate mitotic regulatory mechanisms in which Aurora b and Nek2a kinases orchestrate a faithful chromosome attachment to spindle microtubule, which prevents chromosome instability during cell division. However, it has remained elusive as how Ndc80 complex is assembled and loaded to centromere in mitosis. Using quantitative FRAP and photoactivation localization analysis, we analyzed the dynamics of Hec1, Nuf2, Spc24 and Spc25 proteins in living cells to obtain insight into the dynamics of kinetochore assembly and microtubule attachments throughout the cell cycle. In addition, we employed a new covalent fluorescent pulse-chase labeling approach to delineate how Ndc80 components are assembled and partitioned to centromeres during cell divisions. Surprisingly, assembly of new Ndc80 complex is achieved in prophase in two steps, in which Spc24/25 heterodimer is loaded onto kinetochore prior to Hec1/Nuf2 heterodimer. Using fluorescence resonance energy transfer-based sensors in living HeLa cells, we are current analyzing how Ndc80 tetramer complex assembly is regulated in cell cycle.

2726/B437

Metaphase Functions of Cytoplasmic Dynein: Roles in Stable Kinetochore-Microtubule Attachment, Orientation, and Oscillation.

D. Varma, P. Monzo, S. Stehman, R. Vallee; Pathology and Cell Biology, Columbia University, New York, NY

Cytoplasmic dynein is one of only two force-producing motor proteins at the kinetochore. The kinetochore dynein phenotype has been difficult to define completely because of the multiplicity of mitotic dynein roles, and the use of surrogate genes to address dynein function. Dynein has been implicated in early prometaphase poleward chromosome movement and subsequently in the removal of mitotic checkpoint proteins. However, additional functions have now been suggested by recent studies in our lab using dynein-specific probes, including dynein heavy chain tail fragments, dynein RNAi, and a function-blocking antibody. We find dynein tail fragments to displace the full-length motor-containing dynein heavy chain from kinetochores. 28% of cells exhibit bipolar spindles, and 60% of these cells arrest at late-prometaphase/metaphase, with retention at kinetochores of dynactin, CLASP, Hec1, BubR1, and Mad1. Congression of chromosomes to the metaphase plate occurred over a normal time-course, but many kinetochore pairs were incompletely aligned and/or misoriented relative to the spindle equator. Cold treatment caused massive loss of kinetochore microtubule bundles in these cells, as well as in cells subjected to dynein heavy chain RNAi or antibody injection. This was not due to accumulation of plus end depolymerizing kinesins at kinetochores, as Kif2b and MCAK were normally redistributed to spindle poles. Fixed and live imaging of kinetochores was also performed using cells arrested in metaphase by addition of the proteasome inhibitor MG132. Tail expression or antibody injection caused misorientation and displacement of kinetochore pairs from the metaphase plate, and a striking increase in the amplitude of kinetochore oscillations. These results identify a role for the dynein motor in stable microtubule attachment, and provide evidence for persistent dynein activity at kinetochores during metaphase.
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2727/B438

The Purification of Yeast Kinetochores Identifies a PP1 Regulatory Subunit.

B. Akiyoshi[1], C. Nelson[1], J. Ranish[2], S. Biggins[1]; [1]Fred Hutchinson Cancer Research Center, Seattle, WA, [2]Institute for Systems Biology, Seattle, WA

Chromosomes attach to the mitotic spindle via the kinetochore, the protein complex that assembles on centromeric DNA. Chromosome segregation depends on each pair of sister kinetochores biorienting such that they bind to microtubules from opposite poles. Defects in chromosome biorientation activate the spindle checkpoint, preventing cells from entering anaphase. The conserved Ipl1/Aurora protein kinase is required for biorientation and checkpoint activation because it detects and destabilizes improper microtubule-kinetochore attachments. Ipl1 activity is opposed by Glc7, the sole protein phosphatase I in budding yeast. To understand how cells achieve kinetochore biorientation, it is critical to identify the key regulators and substrates of Ipl1 and Glc7. We therefore developed a method to isolate the budding yeast kinetochore by purifying minichromosomes that contain centromeres. Using this technique, we have detected greater than 95% of known core kinetochore proteins by mass spectrometry (MS) as well as a number of post-translational modifications including Ipl1-dependent phosphorylation. In addition, we found that the Fin1 protein is a previously unknown kinetochore component. Fin1 is known to bind to the yeast 14-3-3 proteins as well as Glc7. The Morgan lab previously showed that CDK1 phosphorylation prevents Fin1 from prematurely localizing to microtubules. We found that the 14-3-3 proteins bind to Fin1 via CDK-mediated phosphorylation. When this interaction is abolished, Glc7 prematurely localizes to spindles in a Fin1-dependent manner. Strikingly, this premature localization of Glc7 leads to a defect in spindle checkpoint activation in response to biorientation defects. We therefore propose that Fin1 is a Glc7 regulatory subunit that dephosphorylates Ipl1 substrates involved in checkpoint activation and its activity is tightly regulated by the yeast 14-3-3 proteins.

Oncogenes and Tumor Supressors (2728 – 2753)

2728/B439

Kinome-Wide Sequencing Studies Provide a Roadmap for the Discovery of a Novel Cancer-Associated Kinase.

J. Brognard, T. Hunter; Molecular and Cell Biology, Salk Institute for Biological Studies, La Jolla, CA

Protein kinases are master regulators of cell growth, proliferation, and cell survival and it is well established that mutated kinases play a casual role in many forms of cancer. Unbiased kinome-wide sequencing studies underscore the complexity of the cumulative mutational changes occurring in cancer, and highlight the possibility that many kinases not previously predicted to play a role in cancer will be important in carcinogenesis. In an evaluation of 518 kinases in 210 cancers, the Sanger Centre group identified several protein kinases predicted to possess driver (i.e. causal) mutations. We have refined their predictions using bioinformatic web applications that take into account evolutionary conservation and the structural consequences of the nonsynonymous amino acid changes. Utilization of these bioinformatic web applications has pinpointed DAPK3 (ZIPK) as a potential tumor-suppressing kinase. Evaluation of nonsynonymous point mutations, discovered in this kinase in various tumors, reveal that 2 out of three point mutations abolish kinase activity. Transient overexpression of wild type DAPK3 causes an increase in MLC2 phosphorylation, however we do not observe an increase in MLC2 phosphorylation when we overexpress two mutants observed in cancer, D161N and P216S. Consistent with this result, incubation of immunoprecipatated wild type DAPK3 with recombinant MLC2 results in an increase in MLC2 phosphorylation in an in vitro kinase assay, whereas the D161N and P216S mutants lack kinase activity. Furthermore, phenotypic assays indicate that all three mutations observed in cancer abrogate the function of the wild type kinase. Transient overexpression of wild type DAPK3 causes an increase in the percent of cells in the G1 phase of the cell cycle, under high serum conditions, and an increase in apoptosis under low serum conditions. Overexpression of the three mutants observed in cancer does not alter either cell cycle progression or cell survival. These data suggest that DAPK3 normally functions to inhibit cell cycle progression and promote apoptosis, and that loss of function mutations in DAPK3 may promote cell cycle progression and cell survival, establishing this kinase as a potential tumor suppressor.

2729/B440

An RNAi Synthetic Interaction Screen Identifies a Link between TP53 and SnRNP Biogenesis Pathway.

D. B. Krastev[1], A. Heninger[2], M. Slabicki[1], M. Paszkowski-Rogacz[1], M. Junqueira[1], F. Buchholz[1]; [1]Max-Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany, [2]Center for Regenerative Therapies Dresden, Dresden, Germany

We have used a genome-scale enzymatically prepared siRNA (esiRNA) library in search for genetic interactions with the TP53 pathway. We compared the growth rates of two cell lines that differ only in their TP53 status in conjunction with depletion of gene function by RNAi. As expected, the majority of hits give a disadvantage to the cells in which TP53 is present. However, several knockdowns showed slower growth in TP53 deleted cells, indicating a potential synthetic interaction. We have focused on a couple of genes whose knock down specifically ceases TP53 knockout mutant's proliferation. To gain insight in the molecular functions of these genes we undertook a proteomic approach. They were expressed at endogenous levels from bacterial artificial chromosomes (BAC) and tagged with a localization and affinity purification (LAP) cassette, which allows for in vivo localization and tandem affinity purification. The in vivo complexes were purified under mild conditions and the protein composition was investigated my mass-spectrometric (MS) analysis. We found an unexpected interplay between TP53 status of the cell and the snRNP biogenesis pathway. Depending on the TP53 status of the cell and depletion of synthetic lethal hits, dramatic alterations in the cellular distribution of snRNP assembly machineries are observed.

2730/B441

Battle of the Sexes: the Roles of TSPY and TSPX in Cell Proliferation and Tumorigenesis.

Y. Li, Y. Lau; Department of Medicine-VAMC, University of California San Francisco, San Francisco, CA

The testis specific protein Y-encoded (TSPY) gene serves vital functions in stem germ cell proliferation in embryonic testis and spermatogonial cells in adult testis. Ectopic expression of TSPY results in accelerated cell proliferation, particularly shortening of the G2/M stage. TSPY is the putative gene for the gonadoblastoma locus on the human Y chromosome (GBY). TSPY is abundantly expressed in tumor cells of gonadoblastoma, testicular premalignant precursors carcinoma-in-situ and seminoma, and various somatic cancers. TSPY and its X-homologue, TSPX, harbor a conserved domain, designated as SET/NAP domain, but differ at their carboxyl termini. Interestingly, over-expression of TSPX retards cell cycle progression at G2/M stage. To explore the molecular mechanisms responsible for such contrasting properties of these SET/NAP domain-containing proteins encoded by the sex chromosomes, we have analyzed their possible interactions with cell cycle regulators at G2/M stage. Our results demonstrated that TSPY and TSPX bind competitively to the mitotic cyclin B at their SET/NAP domains in vitro and in vivo. TSPY colocalizes with cyclin B1 during the cell cycle, particularly on the mitotic spindles at metaphase. TSPY enhances while TSPX represses the cyclin B1-CDK1 phosphorylation activity. The inhibitory effect(s) of TSPX on the cyclin B1-CDK1 complex has been mapped to its carboxyl acidic domain that is absent in TSPY, suggesting that TSPX could serve a normal or tumor suppressor function(s) in modulating cell cycle progression at the G2/M stage while TSPY has acquired a specialized function(s) in germ cell proliferation and meiotic division. The present study suggests that epigenetic dysregulation of TSPY in incompatible germ or somatic cells could promote cell proliferation and predispose susceptible cells to tumorigenesis.

2731/B442

Repression of P53 Activity by Cabin1.

H. Jang, H. Youn; Seoul National University Colleage of Medicine, Seoul, South Korea

The tumor suppressor p53 is known to function primarily as a transcriptional activator by binding to target promoters under cellular stresses. However recent chromatin immunoprecipitation data show that p53 can occupy a subset of its target promoters even in the absence of genotoxic stresses. This observation suggests the requirement of negative regulators of p53, which bind p53 in the absence of genotoxic stresses. Here we show that a calcineurin binding protein 1 (Cabin1) binds p53 and represses the function of p53. Cabin1 is known as a

calcineurin inhibitor and a transcriptional corepressor, which represses myocyte enhancer factor 2 by recruiting various histone modifying enzymes. However previously known function of Cabin1 cannot explain the embryonic lethality of Cabin1 knockout mice. Thus we attempted to find other transcription factors regulated by Cabin1 and found p53 bound to N-terminal region of Cabin1 at amino acid 500-900. In the presence of genotoxic stresses, the protein level of Cabin1 decreased, thereby abrogated the interaction with p53. Overexpression of Cabin1 repressed p53 transcriptional activity in p53 targeting promoters. Knockdown of Cabin1 by short hairpin RNA unregulated mRNAs of a number of p53 target genes. As a consequence, Cabin1 down-regulated cells showed growth retardation and increased cell death upon genotoxic stresses in p53 dependent manner.

2732/B443

Activation of MOB as Tumor Suppressor by Membrane Association in Growth Inhibition.

L. Ho[1], X. Wei[2], T. Shimizu[3], Z. Lai[1,2,4]; [1]Integrative Biosciences Graduate Degree Program, The Pennsylvania State University, University Park, PA, [2]Intercollege Program in Genetics, The Pennsylvania State University, University Park, PA, [3]Department of Biochemistry and Molecular Biology, The Pennsylvania State University, University Park, PA, [4]Department of Biology, The Pennsylvania State University, University Park, PA

Tissue growth and organ size are coordinated by regulating cell proliferation and apoptosis during development. Hippo signaling pathway has been shown to restrict cell proliferation and promote apoptosis. Previous studies have demonstrated that Mob as tumor suppressor protein, Mats, functions as an activating cofactor of Warts (Wts)/large tumor suppressor (Lats) in this pathway; however, ectopic expression of Mats alone is not sufficient to disrupt normal development in *Drosophila*. Recently, human Mps1-One binder (MOB1), a human ortholog of Mats, has been shown to increase human Lats 1 kinase activity at the plasma membrane in mammalian cells. Here we generate myristoylated Mats (myrMats) to allow Mats association onto the plasma membrane in developing *Drosophila* tissues. We found that expression of myrMats causes growth inhibition effects by increasing apoptosis and decreasing proliferation. These effects are mediated by recruiting Wts onto the plasma membrane and increasing Wts kinase activity. Mats-binding defective Wts fails to be associated and activated by myrMats. In addition, epistatic analysis shows that these growth inhibition effects of myrMats require Wts. Our results provide evidence that membrane association of Mats is crucial in activating Hippo signaling pathway for growth inhibition of developing tissues.

2733/B444

Tumor Supressor ARF Promotes Non-Classic Proteasomal Independent Polyubiquitination of COMMD1.

H. Li, Y. Huang; Nanyang Technological University Singapore, Singapore

Although tumor suppressor ARF is generally accepted for its essential role in activating p53 pathway, some of its p53 independent function has also been proposed. Here we report that ARF associates with copper metabolism gene MURR1 domain containing protein 1 (COMMD1) and promotes K63 mediated polyubiquitination of COMMD1 in a p53 independent manner. We found that ARF interacts with COMMD1 in vivo. Deletion analysis of ARF suggested that N-terminal amino acids 15-45 are important for its interaction with COMMD1. In addition, we found that endogenous ARF redistributes from the nucleolus to nucleoplasm and interacts with COMMD1 when DNA is damaged by actinomycin D. Interestingly, we found that ARF promotes polyubiquitination of COMMD1 through K63 of ubiquitin but not K48, which does not target COMMD1 for proteasome dependent proteolysis. Moreover, ARF mutants lacking the interacting domain with COMMD1 did not promote COMMD1 polyubiquitination, indicating that physical association is a prerequisite condition for the polyubiquitination process. Together, these data suggest that the ability to promote K63 mediated polyubiquitination of COMMD1 is a novel property of ARF independent to p53.

2734/B445

Identification of NTKL as a Novel Interacting Protein of MSP58.

C. Hsu[2], D. Lin[1,2]; [1]Institute of Biosignal Transduction, Tainan, Taiwan, [2]Department of Pharmacology, National Cheng-Kung University, Tainan, Taiwan

MSP58/MCRS1, a forkhead-associated domain containing protein, have been implicated in regulation of gene transcription and translation as well as in malignant transformation. It has been reported to interact with several proteins, such as nucleolus protein p120, Mi-2β; transcription factors STRA13, Daxx, and RNA-binding protein FMR. In addition, MSP58/MCRS1 can be localized to the centrosome and forms a complex with centrosomal proteins including Nde1, Su48 and DIPA . To determine the mechanism underling MSP58 involvment in cellular transformation, we employed the yeast two-hybrid screening method to identify novel MSP58-interacting molecules recently. One of positive clone carries cDNA that encodes the central region of NTKL (N-terminal kinase like). NTKL is located on 11q13 and is mapped around chromosomal breakpoints found in several carcinomas, suggesting that NTKL dysfunction may be involved in carcinogenesis. Very little is known regarding the function of NTKL. Endogenous NTKL protein is localized to the centrosome during mitosis. Centrosome abnormalities are associated with progression of aneuploidy and malignant transformation. The finding of the physical linkage between MSP58/MCRS1 and NTKL raises the possibility that deregulation of MSP58/MCRS1 may contribute to cellular transformation by causing centrosomal dysfunction. Therefore, to analyze the role of MSP58/NTKL interaction in centrosome functions would be an important question to be explored.

2735/B446

Molecular Mechanism of MYC Oncogene Addiction in Ovarian Cancer.

T. Prathapam[1], A. Aleshin[1], Y. Guan[2,3], J. W. Gray[2,3], G. Martin[1]; [1]Department of Molecular and Cell Biology, University of California, Berkeley, CA, [2]Department of Laboratory Medicine, University of California, San Francisco, CA, [3]Life Science Division, Lawrence Berkeley National Laboratory, Berkeley, CA

Ovarian cancer, although second in incidence as a gynaecologic cancer, causes more deaths than all other gynaecologic cancers combined. Currently, ovarian cancer ranks fifth in both cancer incidence and mortality among women in the United States. Unfortunately, the pathogenesis of this disease is poorly understood. MYC is amplified in 30-60% of human ovarian tumors, which suggests that MYC may

play a role in the initiation or maintenance of ovarian tumors. Expression levels of MYC were highly correlated with genome copy numbers of the 8q24 amplicon in ovarian cell lines. The functional significance of MYC amplification was assessed by RNAi inhibition. Strikingly, inhibition of MYC inhibited cell proliferation, as determined by BrdU labeling, only in lines with amplified MYC, and not in lines without amplified MYC. We also observed that upon MYC RNAi knockdown, expression of the cyclin-dependent kinase inhibitors p27Kip1 and p21Cip1 is upregulated in MYC-amplified cells, but not in non-amplified cells; cells in which both p27Kip and MYC were subjected to RNAi inhibition were able to enter S-phase. MYC non-amplified cells displayed elevated expression of other MYC isoforms that rendered their proliferation independent of MYC, but siRNA inhibition of all MYC isoforms in MYC non-amplified ovarian cancer cells also inhibited cell proliferation. In contrast proliferation of telomerase-immortalized ovarian surface epithelial cells were not inhibited by siRNA inhibition of all MYC isoforms. Our findings suggest that ovarian cancer cells are addicted to members of the MYC family for cell proliferation and that growth of ovarian cancers may be blocked by MYC inhibition.

2736/B447

Regulation of Ras-Dependent Growth and Proliferation by the Ubiquitin Pathway Downstream of RTK Endocytosis.

C. M. Pfleger, H. Yan, E. A. Horvath; Oncological Sciences, The Mount Sinai School of Medicine, New York, NY

Ras signaling can promote proliferation, cell survival, and differentiation. Mutations in components of the Ras pathway are found in a number of solid tumors and are associated with developmental disorders such as Noonan's syndrome. We have recently discovered that Drosophila tissues containing hypomorphic mutations in E1, the most upstream enzyme in the Ubiquitin Pathway, display cell-autonomous up-regulation of Ras/ERK activity and Ras-dependent overgrowth and ectopic proliferation. The Ubiquitin Pathway is an important regulatory pathway that modifies substrate proteins by attaching the small molecule ubiquitin. Conjugation of ubiquitin, either singly or in a polyubiquitin chain, can direct a protein to different fates including endocytosis or protein degradation. Ubiquitin-mediated regulation upstream of Ras is widely accepted to regulate Receptor Tyrosine Kinase (RTK) endocytosis. However, although the ectopic proliferation in E1 hypomorphic mutants is dramatically suppressed by removing one copy of Ras, removal of more upstream components such as Egfr, the Grb2 homolog drk, or sos shows no suppression. In contrast, other cases of RTK activation (upstream of Ras) can be suppressed by removal of the same upstream components. This could mean that decreased ubiquitination leads to growth-relevant Ras/ERK activation by failing to regulate a step downstream of RTK endocytosis. Indeed, we demonstrate that Drosophila Ras is ubiquitinated, consistent with a recent report of mammalian H-Ras ubiquitination in vitro. Our findings suggest a model that ubiquitination of Ras restricts growth and proliferation. Intriguingly, complete inactivation of E1 in a mosaic fly leads to cell lethality and the non-cell autonomous activation of Ras/ERK in adjacent tissue mimicking oncogenic Ras over-expression. Our findings demonstrate that maintaining sufficient ubiquitin pathway function is required for proper Ras regulation both cell autonomously and non-cell autonomously to prevent inappropriate Ras/ERK-dependent growth and proliferation in vivo and may implicate loss of Ras ubiquitination in developmental disorders and cancer.

2737/B448

Oncogenic B-RAF Negatively Regulates the Tumor Suppressor LKB1 to Promote Melanoma Cell Proliferation.

B. Zheng[2,1], L. Cantley[2,1]; [1]Systems Biology, Harvard Medical School, Boston, MA, [2]Cancer Center, BIDMC, Boston, MA

The tumor suppressor LKB1 - AMPK signaling pathway serves as a critical cellular sensor coupling energy homeostasis to cell growth, proliferation and survival. However, how tumor cells suppress this signaling pathway to gain growth advantage under conditions of energy stress is largely unknown. Here, we show that AMPK activation is suppressed in melanoma cells with the B-RAF V600E mutation and that down-regulation of B-RAF signaling activates AMPK. We find that in these cells LKB1 is phosphorylated by ERK and Rsk, two kinases downstream of B-RAF, and that this phosphorylation compromises the ability of LKB1 to bind and activate AMPK. Furthermore, expression of a phosphorylation-deficient mutant of LKB1 allows activation of AMPK and inhibits melanoma cell proliferation and anchorage-independent cell growth. Our findings provide a molecular linkage between the LKB1-AMPK and the RAF-MEK-ERK pathways and suggest that suppression of LKB1 function by B-RAF V600E plays an important role in B-RAF V600E-driven tumorigenesis.

2738/B449

BLNK Suppresses pre-B Cell Leukemogenesis through Inhibition of JAK3.

J. Nakayama[1], M. Yamamoto[1], K. Hayashi[1], K. Bundo[1], M. Kubo[2], R. Goitsuka[1], M. Farrar[3], D. Kitamura[1]; [1]Division of Molecular Biology, Research Institute for Biological Sciences, Tokyo University of Science, Noda, Japan, [2]Laboratory for Signal Network, Research Center for Allergy and Immunology, RIKEN Yokohama Institute, Kanagawa, Japan, [3]Department of Laboratory Medicine and Pathology, Center for Immunology, The Cancer Center, University of Minnesota, Minneapolis, MN

Pre-B cell leukemia spontaneously develops in BLNK-deficient mice, and pre-B acute lymphoblastic leukemia cells in children often lack BLNK protein expression, demonstrating that BLNK functions as a tumor suppressor. However the mechanism by which BLNK suppresses pre-B leukemia, as well as the identification of other genetic alterations that collaborate with BLNK deficiency to cause leukemogenesis are still unknown. Here we demonstrate that the JAK3/STAT5 signaling pathway is constitutively activated in pre-B leukemia cells derived from BLNK-/- mice, mostly due to autocrine production of IL-7. Inhibition of IL-7R signaling or JAK3/STAT5 activity resulted in the induction of p27kip1 expression and cell cycle arrest accompanied by apoptosis in the leukemia cells. Transgene-derived constitutively active STAT5 (STAT5b-CA) strongly synergized with the loss of BLNK to initiate leukemia in vivo. In the leukemia cells, exogenously expressed BLNK inhibited autocrine JAK3/STAT5 signaling, resulting in p27kip1 induction, cell-cycle arrest and apoptosis. BLNK-inhibition of JAK3 was dependent on the binding of BLNK to JAK3. These data indicate that BLNK normally regulates IL-7-dependent proliferation and survival of pre-B cells through direct inhibition of JAK3. Thus, somatic loss of BLNK and concomitant mutations leading to constitutive activation of JAK/STAT5 pathway result in the generation of pre-B cell leukemia.

2739/B450

Phosphorylation of eiF4E by MNKs Supports Protein Synthesis, Cell Cycle Progression and Proliferation in Prostate Cancer Cells.

A. Bianchini[1], M. Loiarro[1], P. Bielli[1], R. Busà[1,2], M. Paronetto[1,2], F. Loreni[1], R. Geremia[1], C. Sette[1,2]; [1]Public Health and Cell Biology, University of Rome Tor Vergata, Rome, Italy, [2]Laboratory of Neuroembryology, Fondazione Santa Lucia, Rome, Italy

Deregulation of the PI3K/AKT/mTOR and RAS/MAPK/MNK pathways frequently occurs in human prostate carcinomas (PCas) and leads to aberrant modulation of mRNA translation. We have investigated the relative contribution of these pathways to translational regulation and proliferation of PCa cells. MNK-dependent phosphorylation of eIF4E is elevated in DU145 cells, which have low basal levels of AKT/mTOR activity due to the expression of the tumor suppressor PTEN. By contrast, eIF4E phosphorylation is low in PC3 cells with mutated PTEN and constitutively active AKT/mTOR pathway, but it can be strongly induced through inhibition of mTOR activity by rapamycin or serum depletion. Remarkably, we found that inhibition of MNKs strongly reduced the polysomal recruitment of TOP mRNAs, which are known targets of mTOR-dependent translational control. Pull-down assays of the eIF4F complex indicated that translation initiation was differently affected by inhibition of MNKs and mTOR. In addition, concomitant treatment with MNK-inhibitor and rapamycin exerted additive effects on polysomal recruitment of TOP mRNAs and protein synthesis. The MNK-inhibitor was more effective than rapamycin in blocking proliferation of PTEN-expressing cells, whereas combination of the two inhibitors suppressed cell cycle progression in both cell lines. Microarray analysis showed that MNK affected translation of mRNAs involved in cell cycle progression. Thus, our results indicate that a balance between the activity of the AKT/mTOR and the MAPK/MNK pathway in PCa cells maintains a defined translation level of specific mRNAs required for ribosome biogenesis, cell proliferation and stress response and might confer to these cells the ability to overcome negative insults.

2740/B451

Three DNA Binding Assays Reveal Differences in p53 Mutant Protein Binding.

U. Chandrachud, S. Gal; Biological Sciences, Binghamton University, Binghamton, NY

There are multiple assays to measure the DNA binding of transcription factors that are important for correct functioning of cells. We are using electrophoretic mobility shift assays (EMSAs), an assay using streptavidin magnetic beads with biotinylated DNA (SA) and scintillation proximity assay (SPA) beads to analyze the DNA binding of the tumor suppressor p53. Mutations in p53 often change amino acids in the protein's DNA binding domain and are associated with cancer. In this research, we are probing the DNA binding properties of the p53 protein in 4 human cell lines with several different gene sequences. The p53 protein is derived from nuclear extracts of three thyroid cancer cell lines that have different p53 mutations (ARO, WRO and NPA), and a breast cancer cell line with wild-type p53 (MCF-7) treated with hydrogen peroxide. The DNA sequences for binding are derived from the cyclin G, mdm-2 and p21 genes. Treatment of MCF-7 cells with hydrogen peroxide increases the binding of p53 to DNA as detected using the EMSA and SA, but not by the SPA. The mutant p53s have comparable binding reactions in SPA and EMSA. The SA could not detect significant binding of the p53 from the WRO cells to any sequence. The p53 from ARO cells did not bind well to a mutant cyclin G sequence, while the protein from WRO and NPA cells did not discriminate between the 4 sequences. Having these different assays allows us to quantitatively distinguish binding reactions using extracts with wild-type and mutant p53 proteins. We feel the SPA gives a real time analysis of DNA binding and is more quantitative than the other two assays, while the SA allows us to co-purify proteins associated with the DNA bound p53. Both of these assays will allow us to understand interactions that may change in different cells and tumors to affect the function of this important tumor suppressor.

2741/B452

The POZ-ZF Transcription Factor Kaiso Converges on the HIF1-A Signaling Pathway.

K. K. Nanan, A. Thawer, L. Beatty, C. Pierre, J. Daniel; Biology, McMaster University, Hamilton, ON, Canada

A hypoxic tumor center is characteristic of many solid tumors, often leading to poor patient prognosis. The master oxygen sensing mechanism of the cell is HIF1, a nuclear transcription factor consisting of the constitutively expressed HIF1-β and the oxygen-sensitive HIF1-α. Under normoxic conditions, HIF1-α is marked for proteasomal degradation by von Hippel Lindau (VHL) after hydroxylation by prolyl hydroxylase domain-containing enzymes (PHD). Conversely, in the absence of oxygen, HIF1-α is stabilized and the HIF-1α/β heterodimer translocates to the nucleus where it controls the expression of various target genes that enable the cell to cope with hypoxic conditions. Much research has been conducted to elucidate the role of HIF1 in cancer and development but the mechanism of hypoxia-induced changes in gene expression remains to be fully understood. We now have data that implicates the POZ-ZF transcription factor Kaiso in the HIF1 pathway. Kaiso is a nuclear transcriptional repressor initially identified as an interaction partner for the p120-catenin component of the cadherin-catenin cell adhesion complex. In a recent study, it was found that Kaiso expression and subcellular localization was dependent on the tumor microenvironment and this finding led to the hypothesis that Kaiso expression and/or function is regulated by hypoxia. Here we report that Kaiso expression is decreased in cultured cells after chronic hypoxia. We also noticed a shift in Kaiso subcellular localization from the nucleus to the cytoplasm in response to hypoxia. Using qRT-PCR, we detected a decrease in HIF1-α mRNA levels in stable Kaiso overexpression cell lines as compared to parental cells. Finally, a bioinformatics analysis revealed a number of Kaiso Binding Sites (KBSs) in the HIF1-α promoter, and using (electrophoretic mobility shift assay) EMSA, we found that Kaiso specifically binds the HIF1-α promoter KBS sequences. These findings link Kaiso to the HIF1-α signaling pathway and implicate HIF1-α as a potential Kaiso target gene.

2742/B453

Subcellular Distribution of Prohibitin in MCF7 Cells Treated with Tamoxifen.

V. Agrawal, R. Chen, S. Chowdhary, D. Kour, J. McClung; Biology, Wartburg College, Waverly, IA

Prohibitin is located in the mitochondria and the nuclei. In the mitochondria, prohibitin acts as a chaperone protein (Nijtmans et al. EMBO J 19:2444-2451, 2000). In the nuclei, prohibitin inhibits cell proliferation by binding to the transcriptional factors E2F and p53 (Wang, et al. Oncogene 18:3501-3510, 1999; Fusaro, et al. 278:47853-47861, 2003). In addition, prohibitin is required for the growth suppression by estrogen antagonists such as tamoxifen, and estrogen antagonists increase the amount of prohibitin (Wang et al. EMBO J 23:2293-2303, 2004). However, it is not clear if the treatment of tamoxifen changes the subcellular distribution of prohibitin. The distribution of the prohibitin pool between these two compartments may influence prohibitin's function. In this study, immunofluorescent confocal microscopy

was used to determine the distribution of prohibitin between the mitochondria and nuclei in MCF7 cells with or without treatment of tamoxifen. Prohibitin was found to be equally distributed between the mitochondria and nuclei in untreated MCF7 cells. After tamoxifen treatment, the overall prohibitin levels increased slightly but the distribution between mitochondria and nuclei remained equal. Tamoxifen's inhibition of cellular proliferation through prohibitin is not due to changes in the organelle distribution of prohibitin, but is more likely due to changes in prohibitin's association with other factors in the nuclei.

2743/B454

MSP58 Is a Nucleolar Protein Involved in the Regulation of Telomerase Activity.

D. Lin; Institute of Biosignal Transduction, Tainan, Taiwan

The 58-kDa microspherule protein (MSP58; MCRS1) has been reported to interact with several proteins, such as nucleolus protein p120, Mi-2β, transcription factors STRA13, Daxx, and RNA-binding protein FMR. These findings suggest that MSP58 may have functions of transcriptional regulation in the nucleus and nucleolus. A study of the quail homologue of MSP58, TOJ3, showed that this protein exhibits cellular transformation potential. Moreover, a study revealed that MSP58 behaves as an oncogene and that its transformation activity can be inhibited by physical interaction with PTEN tumor suppressor. Recently, a splice isoform of human MSP58, MCRS2, was found to interact with potent telomerase inhibitor LPTS/PinX1, and forced expression of MCRS2 resulted shortening of telomeres. To determine whether MSP58 has an effect on telomerase activity, MSP58 plasmid was satbly transfected into HT1080 cells, a telomerase-positive fibrosarcoma cell line. The growth of stable MSP58 cells apparently slowed down at early passages. Importantly, MSP58 stably expressed clones showed significantly reduced telomerase activities compared to the corresponding control or parental cells. Immunofluorescence showed that MSP58 localized in the nucleus, especially in nucleoli and weakly at telomere speckles. Further studies are required to understand the roles of MSP58 in the regulation of cell proliferation and telomerase activity .

2744/B455

Function and Regulation of PML Nuclear Bodies.

K. Keusekotten[1,2], S. R. Weisshaar[1,2], A. Krause[1,2], H. M. Springer[1,2], C. Horst[1,2], K. Göttsche[2], R. Dohmen[2], G. Praefcke[1,2]; [1]Junior Research Group 6, Center for Molecular Medicine Cologne, Cologne, Germany, [2]Institute for Genetics, University of Cologne, Cologne, Germany

Promyelocytic leukemia (PML) nuclear bodies (NBs) are multiprotein complexes involved in various processes ranging from an active role in transcription, DNA repair, DNA replication and RNA transport to a role as intranuclear protein depots. PML-NBs are defined by the presence of the PML protein which is essential for their formation. In patients suffering from acute promyelocytic leukemia, the PML gene is fused to the gene for the retinoic acid receptor α due to a chromosomal translocation and PML NBs are disrupted. Treatment with arsenic trioxide (ATO) leads to the degradation of the fusion protein and results in remission of the disease. The assembly of PML-NBs is regulated by the attachment of the small ubiquitin-like modifier (SUMO) protein to PML and other PML-associated proteins such Sp100 and Daxx and creates binding sites for other proteins containing short SUMO interaction motifs (SIMs). We have recently reported that poly-SUMO conjugates are subject to a ubiquitin-dependent proteolytic control in yeast and human cells mediated by a novel class of specific ubiquitin-ligases for SUMO conjugates (ULS) containing a RING domain and several SIMs. Here we show that ATO induces a stress response leading to an increase of SUMO-2/3 conjugates. The elevated levels of modified PML lead to its subsequent ubiquitylation. The SUMO-binding ubiquitin ligase RNF4 mediates this modification and causes disruption of PML nuclear bodies upon treatment with ATO. Reconstitution of SUMO-dependent ubiquitylation of PML by RNF4 in vitro and in a yeast trans vivo system reveals a preference of RNF4 for chain forming SUMOs. In summary, the molecular mechanism for the therapeutic effect of ATO is mediated by poly-sumoylation of PML leading to its recognition and ubiquitylation by the ULS protein RNF4.

2745/B456

Defect in Tumor Suppressor p53 Is Associated with Upregulation of Human Phosphoserine Aminotransferase (PSAT) Gene Expression in Tumor Cells.

Y. Kim, D. Jun, J. Lee, H. Park; School of Life Sciences and Biotechnology, Kyungpook National University, Daegu, South Korea

Phosphoserine aminotransferase (PSAT; EC 2.6.1.52) catalyses the conversion of 3-phosphohydroxypyruvate into 3-phosphoserine, which is the second enzymic step in the phosphorylated pathway of L-serine biosynthesis. Up-regulation of PSAT gene has been reported in many tumor cells, and considered to confer a growth advantage to tumor cells due to the metabolic importance of L-serine for nucleotide synthesis directly linked to cellular replication. Recently, it has been suggested that the PSAT over-expression may result in cancer development and chemoresistance in breast and colon cancer. Until now, however, a contribution of the transcriptional dysregulation for the PSAT-upregulation associated with human neoplasia remains largely unknown, due to the unavailability of the information regarding transcriptional regulation of PSAT promoter. In this context, we have cloned the promoter region of the human PSAT gene by polymerase chain reaction and have characterized its transcriptional regulation mechanism. In this study, we show that repression of the PSAT promoter was mediated by the wild-type p53 protein through binding of consensus p53 cis-element in the PSAT promoter, but not by the tumor-derived mutant forms. In addition, we elucidate that the wild-type p53 protein can repress the expression of PSAT by binding directly with the positive regulators, such as Sp1 and NF-Y, of the PSAT transcription machinery. Together these results demonstrate that the defect of p53 protein resulting from mutation is a responsible mechanism for the abnormal upregulation of the PSAT gene in many tumor cells.

2746/B457

A Novel Adhesion Molecule Bves Alters Colon Cancer Cell Adhesion, Migration and Proliferation.

J. Yang[1], C. S. Williams[2], S. Presley[1], M. S. Chang[1]; [1]Ophthalmology, Vanderbilt Univ, Nashville, TN, [2]Medicine, Vanderbilt University, Nashville, TN

Bves (blood vessel/epicardial substance) is a transmembrane protein that belongs to the popdc gene family and first discovered as an enriched gene product in the developing heart. We have demonstrated that Bves regulates epithelial-mesenchymal transition (EMT) in human cornea epithelial cells and is essential in regulating tight junction (TJ) formation in various epithelial cell types. EMT has been recognized to play

pivotal roles in embryonic development and in tumor growth and invasion. In the present study, we found that over-expression of Bves alters LIM2405 cancer cell motility. Boyden chamber assays show migration and invasiveness of these cancer cells are decreased. In addition, increased Bves expression also reduces cellular proliferation and prolongs cell doubling time by approximately 35%. By flow-cytometry, we found that the over-expressing Bves in LIM2405 cells reduces the percentage of S phase compared to parental LIM2405 cells. This S phase reduction becomes more apparent with increasing confluence, suggesting that contact inhibition is restored with over-expression of Bves. These effects of Bves may be partially through tight junction associated Rho signaling and transcription, since Bves regulates TJ formation. TJs alter Rho signaling by sequestering GEF-H1, an activator of RhoA. Similarly, TJs regulate gene transcription by sequestering a y-box transcriptional factor, ZONAB/DbpA. LIM2405 cells over-expressing Bves exhibit increased TJ formation, along with decreased RhoA activation and ZONAB/DbpA transcriptional activity. Our data demonstrate for the first time that Bves, a novel molecule, may play an important role in regulating colon cancer cell adhesion, migration and proliferation. These findings warrant further investigation to determine Bves' role in cancer biology and may lead to new therapies.

2747/B458

The Bi-Cistronic Mirna Cluster, let-7a3-b, Is Downregulated in Human Colon Carcinomas and Inhibits Oncogenic Target mRNAs, Ras Activity and Signaling, Cell Proliferation, and Anchorage-Independent Growth.

P. S. Mongroo, C. King, C. Johnstone, M. Bowser, S. Rich, A. Rustgi; Department of Medicine, Gastroenterology Division, University of Pennsylvania, Philadelphia, PA

Colorectal cancer (CRC) has served as a unique platform for durable insights into and advances in molecular pathogenesis, chemoprevention, diagnosis, and therapy, not only in this venue but expanded into other disease states. It is well accepted that the majority of sporadic colorectal cancers features the chromosomal instability pathway, involving alterations in key tumor suppressor genes (APC and p53, but also SMAD4) and oncogenes (especially Ki-Ras, but also EGFR, c-myc, B-Raf) and their downstream effectors. A subset of sporadic colon cancers undergo microsatellite instability (MSI) pathway, typically right-sided with B-Raf mutations but without Ras mutations. We now have discovered a new telomeric region of loss of hertozygosity (LOH) on chromosome 22q13, which harbors two microRNAs (miRNAs), namely let-7a3 and let-7b. Quantitative real-time PCR (qRT-PCR) analysis showed these miRNA are both downregulated in human colon cancer cell lines (CRCs) and up to 40% of colorectal tumors relative to normal colonic epithelium. For functional studies, we stably infected Caco-2 cells, which express low or undetectable levels of endogenous let-7, with a retrovirus constitutively expressing the let-7a3-b cluster or empty vector as a control. Exogenous primary transcript and mature let-7 miRNA expression was confirmed by conventional intronic RT-PCR and qRT-PCR respectively. Using western blot analysis, we found that constitutive expression of let-7a3-b suppresses endogenous RAS protein levels, in addition to two previously established let-7 cell cycle-associated targets, namely HMGA2 and c-myc, relative to vector control cells. Furthermore, let-7a3-b inhibited RAS activation in Raf1-RBD pull-down assays and inhibited both basal and EGF-induced phosphorlyation of the Ras signaling pathway effectors, MEK and MAPK. Importantly, let-7a3-b significantly reduced Caco-2 cell proliferation, and colony size and formation in soft agar assays. Based on these findings we hypothesize that the let-7a3-b microRNA cluster on 22q plays a novel role in colorectal cancer progression, by limiting cell proliferation pathways.

2748/B459

Extracellular Signal Regulated Kinase 8 (ERK8) Is Inactivated in Breast Cancer.

A. Groehler, D. A. Lannigan; Microbiology, University of Virginia, Charlottesville, VA

Extracellular signal Regulated Kinase 8 (ERK8) is a kinase whose function is not well understood, however, ERK8 has been shown to be activated by the overexpression of a number of oncogenes, such as RET/PTC3, Bcr-Abl and c-src (1,2). ERK8 is a member of the ERK1/2 subfamily. ERK8 and ERK1/2 share higher sequence similarity (45%) in the kinase domain, but ERK8 has a very divergent (sequence similarity 5%) and much larger C-terminal tail than ERK1/2. We found that overexpression of ERK8 increased proliferation of MCF10A, a normal breast cell line by ≥ 2-fold. Additionally, the ectopic expression of ERK8 increased the number of MCF10A cells in S phase by more than 3-fold. Silencing of endogenous ERK8 by short hairpin RNA reduces proliferation by 40% and can be rescued by ectopically expressed ERK8, which contains a silent mutation making it resistant to knockdown. Kinase dead and kinase inactive ERK8 were unable to stimulate the proliferation of MCF10A cells but did not act as dominant negatives. Kinase dead ERK8 was unable to translocate to the nucleus, which suggests that kinase activity is important for nuclear import. Because ERK8 drives proliferation in non-transformed breast epithelial cells we tested whether ERK8 would increase proliferation in breast cancer cells. Surprisingly, ectopic expression of ERK8 did not increase proliferation in MCF-7 breast cancer cells. Furthermore, knockdown of ERK8 did not alter proliferation of breast cancer cells. ERK8 was found to be inactive in MCF-7 and T47D breast cancer cell lines, but active in MCF10A, and NMuMG non-transformed mammary epithelial cell lines, and freshly isolated primary human mammary epithelial cells. These data indicate that active ERK8 is required for cell cycle progression in untransformed breast cells but not in breast cancer cells. 1. Abe, M. K. et al. ERK8, a new member of the mitogen-activated protein kinase family. J Biol Chem 277, 16733-43 (2002). 2. Iavarone, C. et al. Activation of the Erk8 mitogen-activated protein (MAP) kinase by RET/PTC3, a constitutively active form of the RET proto-oncogene. J Biol Chem 281, 10567-76 (2006).

2749/B460

Cell Growth Regulation of Ouabain and Methyl-B-Cyclodextrin in Breast Cancer Cells.

L. A. Barbosa[1], L. Liu[2], C. L. Fontes[3]; [1]Departamento Técnico Científico, INCa - Instituto Nacional de Câncer, Rio de Janeiro, Brazil, [2]University of Toledo, Toledo, OH, [3]Leandro Barbosa, Instituto de Bioquímica Médica, Rio de Janeiro, Brazil

Objective: Recently, it was demonstrated that when breast cancer cells MDA-MB-435s were treated with ouabain, the activation of $Scr/ERK_{1/2}$ and increase of expression of p^{21cip1} occured. However, several studies demonstrated that the cellular content of caveolin-1 was related with cell proliferation. Breast cancer cell has a restoration of normal phenotype after the transfection of caveolin-1. In our work, we treated the cells by a long-term fashion with ouabain and with methyl-beta-cyclodextrin (MBC) to evaluate its effects on caveolae and cell growth. Methods: The analysis of caveolae content after treatment with ouabain 100nM for 24h and MBC 10mM for 1h was carried out, we used the sucrose gradient fractionation of cell lysates by carbonate method, following an ultracentrifugation step to obtain the plasma

membrane fraction. We performed immunoblotting experiments using antibodies anti-Na, K-ATPase, Src kinase and caveolin-1. To proliferation assay, we performed cell counts using a Z2 Coulter Counter. The counts were made after ouabain treatment (100nM for 24h and 48h) or MBC treatment (10mM for only 1h) or medium containing ouabain (100nM for 24h or 48h) plus MBC. Results and Conclusions: After the treatment with ouabain and MBC, we observed a redistribuition of caveolae proteins, with a spread out profile, to heavy fractions. It is important to notice that the protein profile suffered non-significant changes from control to cell-treated conditions. Futhermore, the total expression of proteins after ouabain treatment caused a downregulation of only α1-Na, K-ATPase levels. In the proliferation assays we observed the growth arrest effect of ouabain in these cells, and that MBC alone was sufficient to cause growth arrest. Interestingly, when cells were treated with both, MBC plus ouabain, an additional growth arrest effect occurred. Moreover, when we treated the cells with an inhibitor of Src kinase, PP2, we did not observe any effect in ouabain treated cells. Assuming that MBC was a potent depletor of cholesterol in cell membranes, we can presume similar pathways on cholesterol depletion with long-term treatment with ouabain and MBC.

2750/B461

Inhibition of S-Adenosylmethionine Decarboxylase Does Not Prevent Putrescine-Mediated Induction of Spermidine Spermine N1-Acetyltransferase in Mammalian Cells.

A. A. Ancheta[1], L. Hawel[2], C. V. Byus[1,2]; [1]Biochemistry & Molecular Biology, University of California Riverside, Riverside, CA, [2]Biomedical Sciences, University of California Riverside, Riverside, CA

Polyamines are aliphatic cations which are involved in various cellular processes including cell growth and proliferation. Putrescine, spermidine and spermine constitute the major polyamines of the polyamine metabolic pathway. Ornithine decarboxylase (ODC) and spermidine spermine N1-acetyltransferase (SSAT) are the rate-limiting enzymes of the polyamine biosynthesis and polyamine catabolic pathways, respectively. Many studies have shown that alterations in polyamine pools through aberrant regulation of ODC or SSAT may play key roles in disease models including cancer. Our previous research has shown that increasing intracellular putrescine by either inducing high ODC activity or through administration of exogenous putrescine has resulted in elevated SSAT in various mammalian lines, but the mechanism remains unclear. There has been some speculation that the induction of SSAT is mediated through another key enzyme in the polyamine biosynthesis pathway, s-adenosylmethionine decarboxylase (SAMDC). We wanted to determine the effects of blocking SAMDC activity on putrescine-mediated SSAT induction. Our cell lines were treated with the SAMDC inhibitor, SAM486, and/or with exogenous putrescine for a 24 hour period. Intracellular polyamine levels, SSAT and SAMDC enzyme activities were determined. Exogenous administration of SAM486 resulted in elevated intracellular putrescine and acetylspermidine levels with a concomitant decrease in intracellular spermidine and spermine levels. Treatments with SAM486 prevented putrescine-mediated SAMDC induction, but SAM486 proved ineffective in preventing putrescine-mediated SSAT induction. Our results show that inhibition of SAMDC has no effect on putrescine-mediated SSAT induction in our mammalian cell lines.

2751/B462

Rhus Verniciflua Stokes (RVS) Enhances NK Cell Activity and Induces IFN-γ Secretion from Th1-Cells.

E. Lee; Department of East-West Medicine, Graduate School, Kyung Hee University, Seoul, South Korea

Recently, various biological activities of Rhus verniciflua Stokes (RVS) have been reported. For example, an ethanol extract from RVS was found to have an antioxidant effect against hydroxyl radicals and antiproliferative activity against human cancer cell lines. These studies indicate that RVS has an anti-tumour effect, however the exact mechanism of RVS on NK cells and helper T-cells remains to be verified. The purposes of this study was as follows; (1) whether RVS increases NK cell activity, (2) whether RVS increases cytokine expression, such as IFN-γ, TNF-α, IL-4, IL-5 and IL-10, from Th1 and Th2 cells. NK cells and CD4 T-cells were highly purified by using magnetic beads in the spleen of BALB/c mice. CD4 T-cells are polarized to Th1-cells by rIL-12 and anti-IL-4 and Th2-cells by rIL-4 and anti-IL-12. RVS increased NK cell activity directly in Calcein-AM release assay and augment IFN-γ and TNF-α secretion from Th1-cells in ELISA assay. Thus, these results suggest that RVS has a capability for a cancer chemopreventive agent and therapeutic substance.

2753/B464

Molecular Targets of Manganese in Manganese-Exposed PC12 Cells Provide Clues to Plausible Neurodegeneration Mechanism.

R. R. Reams, E. Taka; Pharmacy, Florida A&M University, Tallahassee, FL

Background. Continuous exposure to excessive amounts of Manganese can cause a serious neurological disorder called manganism which can lead to a movement disorder. Manganism, closely resembles Parkinson's disease but differs from PD in that in Manganism, manganese accumulates in the globus pallidus and striatum; however in PD manganese damages dopaminergic neurons. The mechanism of manganese neurotoxicity is still not fully understood, but several studies suggest that Mn neurotoxicity involves disruption of the mitochondria, which leads to MAPK activation, oxidative stress, cytochrome c release and eventual apoptosis. Objective. The objective of this study was to identify the genes targeted by manganese in a model neuronal cell line in order to better understand Mn-induced cell death. Methods. We identified important molecular targets of Mn in rat Pheochromocytoma cells by using microarray gene expression profiling and a commercially available bioinformatic software program.. Results . Mn altered the expression of 223 genes, 146 were up-regulated and 77 were down regulated. Particularly, manganese selectively modulated genes involved in cell cycle, apoptosis, cell proliferation, angiogenesis, hypoxia-responsive, proto-oncogenes and oncogenes as well as genes involve in the immune process. Using real-time PCR, m RNA expression was confirmed for genes involved in cell cycle (cyclin G1, c-Jun, Jun-B, cdk4 and genes involved in apoptosis/anti-apoptosis; btg2, Trp53imp1, Bcl-2, Ho1, Nqo1 and Egln3. Conclusions. These results provide important clues to the interplay of Mn, hypoxic signaling, mitochondrial oxidant signaling and Neurodegeneration of PC12 cells. Acknowledgements: This work was supported by the Association of the Minority Health Professions (AMPHS) and The Agency for Toxic Substances Disease Registry (ATSDR).

Single Molecule Technologies (2754 – 2762)

2754/B466

Mica "Cells" and the Origins of Life: First Steps.

H. G. Hansma, N. S. Fay, M. Murphy, E. M. Petrossian, R. Geller, A. Chworos; University of California, Santa Barbara, CA

The first experiments are in progress to test the hypothesis that life might have originated between mica sheets. The spaces between the 1-nanometer(nm)-thick mineral sheets of mica might have been the precursors of living cells, where the bio-macromolecules for metabolism and replication could evolve in semi-isolated compartments before being encapsulated within lipid membranes. Mica often occurs naturally as 'books,' which are stacks of mica with ~a million sheets per millimeter. Sheets are often centimeters to decimeters long and wide, and mica books can be a centimeter or more thick. Mica's structure is similar to that of illite clays, which do not swell and shrink in response to wetting and drying. Therefore the mica hypothesis has been criticized, because water is necessary for life. Water and aqueous fluids do, however, seep or percolate inward for several millimeters between mica sheets when the sheets are soaked in water or salt solutions for a week or two. We hypothesize that this would provide a good environment for the origins of life, in which some water would be present; but the primordial mica 'cells' would be stable. We have proposed that the spaces between mica sheets reduce the entropy of organic molecules, as compared with the same molecules tethered to a surface or free in a 'prebiotic soup.' This raises the question: how thick are the water layers between mica sheets? Our data show that the thinnest layer of water that forms a continuous fluid layer between 2 mica sheets in air is ~250 nm. Thinner fluid layers are expected when fluid seeps only part way between pairs of mica sheets. Mica has an isoelectric point of 2.2. When 1 gram of powdered mica is suspended in 7 mL purified water, the pH of the aqueous fluid rises ~1.5 pH units. Finally, we are investigating whether mica can catalyze the formose reaction. The formose reaction auto-catalytically produces sugars from formaldehyde in the presence of divalent metal ions. The formose reaction is hypothesized to be a reaction that was important in the origins of life.

2755/B467

Identification, Characterization, and Functional Importance for a Proline Directed STAT5 Serine Kinase.

A. Mitra[1], Z. Nagy[1], H. Rui[2], R. Kirken[1]; [1]Biology, University of Texas at EL Paso, El Paso, TX, [2]Cancer Biology, Thomas Jefferson University, Philadelphia, PA

Hematological malignancies such as leukemia and lymphoma develop from aberrant changes in the molecular pathways that lead to uncontrolled proliferation and differentiation of blood cells. Among different types of blood cells, T lymphocytes are the critical regulator of cell-mediated immunity as well as the peripheral tolerance. Transcriptional regulators orchestrate the expressions of different genes to execute diverse biological functions of T lymphocytes. Aberrant activations of molecular pathways have been mechanistically linked with leukemias, lymphomas and other T cell mediated diseases. JAK (Janus Kinase)-STAT (Signal transducer and activator of transcription) pathway plays crucial roles towards activation, differentiation, interferon response as well as peripheral tolerance of T lymphocytes in response to broad spectrum of cytokines. Constitutive activated STATs have been described in malignancies of T lymphocytes. Growing body of evidence suggests that dysregulated STAT5 has been correlated with the development of cancer in T lymphocytes. Serine phosphorylation (pS) at the transactivation domain of hyperactivated STAT5 has been found in different malignancies. However very little research was performed to investigate the importance as well as to characterize this modification of STAT5. In order to understand the regulatory role of pS at PSP motif of the transactivation domain of STAT5 under Interleukin-2 (IL-2) driven process, we have screened the CMGC (C-Cyclin, M-Mapk, G-Glycogen synthase, C-Clk) kinase family (group of serine/threonine kinases) to identify potential proline directed serine/threonine kinase(s) that can phosphorylate the serine residue of PSP motif of STAT5 transactivation domain in an IL-2 dependent manner. Our preliminary result, based on loss of function approach, suggests towards putative proline directed serine/threonine kinase(s) that can phosphorylate that site of STAT5.Further investigation with kinase(s) and characterization of the site will shed light on suitable therapeutic strategy to regaulate the activity of hyperactivated STAT5 in cell specific manner.

2756/B468

Superresolution Imaging in Live *Caulobacter Crescentus* Cells Using Photoswitchable Enhanced Yellow Fluorescent Protein.

J. S. Biteen[1], M. A. Thompson[1], N. K. Tselentis[1], L. Shapiro[2], W. E. Moerner[1]; [1]Department of Chemistry, Stanford University, Stanford, CA, [2]Department of Cell Biology, Stanford University, Stanford, CA

Recently, photoactivation and photoswitching have been used to control single-molecule fluorescent labels and produce images of cellular structures beyond the optical diffraction limit (e.g., PALM, FPALM, and STORM). In each imaging cycle, only a sparse subset fluorophores is activated and localized, and a final image is generated from the measured single-molecule positions. These imaging strategies are based on the imaging of nanometer-sized single-molecule emitters and on the use of an active control mechanism to produce sparse subensembles. While previous such live-cell studies relied on sophisticated photoactivatable fluorescent proteins, we show here that superresolution imaging can be performed with fusions to the commonly used fluorescent protein EYFP. EYFP has many advantages over other proteins that have been used for live-cell superresolution imaging. Unlike PA-GFP and Dronpa, which must be excited at 488 nm, EYFP can be excited at 514 nm, a redshift that substantially reduces the autofluorescence of the cells, a critical factor in single-molecule cellular imaging. As well, rather than being photoactivated, EYFP can be reactivated many times after apparent photobleaching. Finally, EYFP is a monomeric protein, hence less disruptive than dimeric labels like EosFP. Here, photoinduced reactivation of EYFP is used to produce superresolution images of intracellular proteins in living cells. We address the limitations arising from physiologically imposed upper boundaries on the fluorophore concentration by employing dark timelapse periods to allow single-molecule motions to fill in filamentous structures, increasing the effective labeling concentration while localizing each emitter at most once per resolution-limited spot. We image cell-cycle-dependent superstructures of the bacterial actin homolog MreB in live *Caulobacter crescentus* cells with sub-40-nm resolution for the first time. We then extend our live-cell technique to three dimensions, resolving the midplane ring formed by the prokaryotic cell division protein, FtsZ. These studies show that EYFP is a useful emitter for *In Vivo* superresolution imaging of intracellular structures in bacterial cells.

2757/B469

Effect of Receptor Density on EGF-EGFR Interaction and EGFR Dimerization Kinetics.

C. Yu[2], J. Chen[2], J. Hale[3], K. Ritchie[3], N. K. Prasad[1], J. Irudayaraj[2]; [1]Basic Medical Sciences, Purdue University, West Lafayette, IN, [2]Agricultural Bioengineering, Purdue University, West Lafayette, IN, [3]Physics, Purdue University, West Lafayette, IN

Epidermal growth factor (EGF) has long been a standard model for the study of signaling mechanisms and still remains extremely relevant. An inherently complex EGFR system is better understood by modeling the biological response in quantitative terms. The primary goal of this study is to develop a precise understanding of EGF-EGFR signaling by monitoring surface- and internal events using single molecule methodologies in order to quantitatively predict their inter-relationships in the signaling cascade. As part this task, we analyzed binding kinetics of epidermal growth factor (EGF) to its receptor (EGFR), as a function of EGFR numbers present in a cell, using HeLa (50,000/cell) and MDA-MB-468 (2 million/cell) cells at single molecule level by Total Internal Reflectance Fluorescence (TIRF) microscopy. Mathematical modeling of binding kinetics revealed both similarities and crucial differences between these two cell types. In both cell types, pre-formed EGFR dimers on the cell surface first bind to EGF followed by a conformational change prior to the second EGF binding. However, compared to Hela, MDA-MB-468 cells show; a) higher number of pre-formed dimers, with no major change in dimer to monomer ratio, b) improved EGF-EGFR interaction at lower ligand concentrations, and c) shorter time-lapse between first and second EGF binding to the dimer. AG1478, an EGFR inhibitor, increases both the proportion and the number of pre-formed EGFR dimers while eliminating a vital kinetic intermediate of EGFR dimer between first and second EGF binding. These results demonstrate the strength of single molecule approaches to detect signaling nuances of significant biological importance. Future experiments will focus on integrating phosphorylation events during the signaling by Fluorescence Lifetime Imaging (FLIM) and Correlation Spectroscopy (FCS). Upon integration and further validation, the surface kinetic parameters, such as these and the intracellular events will serve as surrogate markers to predict the biological outcome and enhance our understanding of signaling pathways in vivo.

2758/B470

Modifications of Membrane Protein Diffusion in *P. Falciparum*-Infected Erythrocytes Studied by Quantum Dot-Based Multi-Particle Tracking.

F. Tokumasu[1], M. L. Clarke[2], G. Crivat[1,2], G. R. Ostera[1], J. Hwang[2]; [1]Laboratory of Malaria and Vector Research, National Institutes of Health, Bethesda, MD, [2]Optical Technology Division, Physics Laboratory, National Institute of Standards and Technology, Gaithersburg, MD

Plasmodium falciparum, a deadly form of malaria parasite, induces variety of erythrocyte membrane modifications after invasion. Parasitized erythrocytes show a large number of protrusions, 'knobs,' which include both parasite-derived and erythrocyte membrane proteins. In addition, accumulations of hemichrome, a degradation product of hemoglobin, on the cytoplasmic side of erythrocyte membrane as well as increased band 3 clustering are also characteristic membrane modifications in parasitized erythrocytes. These modifications should dramatically change the host membrane matrix and affect protein diffusions and functions. It is important to study individual protein behavior which is highly impacted by intermolecular interactions and localizations. To study changes in membrane protein diffusion on *P. falciparum*-infected erythrocytes, we developed a multi-protein tracking technique using quantum dots in live cell conditions. To monitor membrane proteins at the single-molecule level, we labeled CD47 with quantum dots and recorded fluorescence video with 1000 frames. Using custom analysis software allowing simultaneous multiple protein tracking, diffusion constants of CD47 were compared between parasitized and unparasitized erythrocytes. CD47 have both mobile and immobile pools. The majority of diffusion constant of CD47 for both parasitized and unparasitized erythrocytes appeared in the <0.5 pixel2/frame range. These molecules are probably in relatively immobile pool due to interactions with Rh-complex and band 3. In parasitized erythrocytes, the population of actively moving protein at above any given cut off value of diffusion constant is smaller than in unparasitized erythrocytes. In particular, the fraction of CD47 in parasitized erythrocytes with diffusion constants above a cut off value range between 2.8~5 pixel^2/frame(≈10-6% of total population) was in half that of CD47 in unparasitized erythrocytes(≈23-12%). Since CD47 are linked to band 3, the known band 3 clustering may be responsible for the reduction of CD47 in mobile pool. These data show that our protein tracking and multi-particle detection techniques can reveal the modifications of diffusion pattern by parasites at video frame rate.

2759/B471

Determination of the Oligomeric State of Protein BAX during Activation in Detergent Micelles Using Single-Molecule Sensetivity Fluorescence Techniques.

O. Ivashyna[1], A. J. García-Sáez[2], J. Ries[2], E. Christenson[1], P. Schwille[2], P. H. Schlesinger[1]; [1]Cell Biology and Physiology, Washington University in St. Louis School of Medicine, St. Louis, MO, [2]BIOTEC, TU Dresden, Dresden, Germany

BAX is a pro-apoptotic member of the BCL-2 protein family. At the onset of apoptosis, monomeric, cytoplasmic BAX is activated and translocates to the outer mitochondrial membrane (OMM). In the OMM BAX participates in formation of an oligomeric pore capable of releasing apoptotic factors into the cytoplasm. Once in the cytoplasm apoptotic factors initiate activation of the effector caspases which execute the cell death process. The biophysical mechanism of BAX activation is controversial and several *in vitro* and *In Vivo* methods of its activation are known. One of the most commonly used *in vitro* methods is activation with non-ionic detergents, such as n-octylglucoside. During BAX activation with non-ionic detergents, BAX interacts with detergent micelles and changes protein conformation from inactive to active. During this activation process BAX forms complexes with detergent micelles whose molecular weight (MW) (250 kDa) is larger than combined MW of BAX monomer (21 kDA) and n-octylglucoside micelle (26 kDa). These high MW complexes have been ascribed to the oligomerization of BAX prior to its membrane insertion and pore formation. This is in contrast with the *In Vivo* studies which suggest active BAX inserts into the OMM as a monomer and then undergoes oligomerization to form a pore. To determine weather BAX activation requires protein oligomerization or whether active BAX can be generated from a protein monomer we have determined the number of BAX proteins per detergent micelle using an approach which combines two single-molecule sensitivity techniques - fluorescence correlation spectroscopy (FCS) and fluorescence-intensity distribution analysis (FIDA). We have tested a range of detergents: n-octylglucoside, dodecyl-maltoside, Triton X-100, Tween 20, CHAPS and cholic acid. With these detergents we consistently observe that BAX is a monomer before, during and after interaction with detergent micelles. We conclude that detergent activation of BAX is not congruent with oligomerization and that in

physiologic buffer conditions BAX can assume two stable monomeric conformations: one inactive and one active. This conclusion is in agreement with the mechanism of BAX induction of apoptosis that is seen in cells.

2760/B472

Vivo-Morpholino Oligomers Induce Potent Exon-Skipping of Dystrophin in Cardiac and Skeletal Muscles of Mice.

S. Jiang, P. A. Morcos; Biology, Gene Tools, LLC, Philomath, OR

Duchene muscular dystrophy (DMD) is a severe muscle disorder caused by frameshift and nonsense mutations in the dystrophin gene. Morpholino antisense oligomers have been shown to induce exon skipping and partially restore the function of dystrophin in the skeletal muscles of animal models of DMD, particularly in mdx mice. However, exon-skipping mediated by morpholino oligomers in the heart is moderate due to reduced delivery efficiency in cardiac muscles. Vivo-Morpholinos, non-peptidic transporter-conjugated morpholino oligomers, exhibit high delivery efficiency in vivo with no detectable toxicity. We examined the efficiency of exon skipping mediated by a Vivo-Morpholino oligomer, V-Mor23, targeting dystrophin exon 23 in normal mice. Intraperitoneal (I.P.) injection of V-Mor23 achieved higher efficiency of exon skipping than intravenous (I.V.) injection in the diaphragm, while I.V. injection achieved more efficient for most other tissues. Two daily I.V. injections of 30 mg/kg V-Mor23 induced potent exon-skipping in the heart (62%), diaphragm (66%) and extensor digitorum longus (70%), which are critical muscles in the treatment of DMD. The effect is dose-dependent and is achieved as rapid as one day after injection. This is the first report of rapid and robust dystrophin exon 23 skipping in the heart of normal mice injected with oligomers. V-Mor23 also exhibits high levels of exon skipping in the cardiac muscles of mdx mice. The high degree of exon skipping achieved in critical muscle types including the heart and diaphragm suggest Vivo-Morpholinos represent a potential treatment for DMD patients.

2761/B473

Transcription Termination Studied at the Single-Molecule level.

M. H. Larson[1], W. J. Greenleaf[2], R. Landick[3], S. M. Block[4,5]; [1]Biophysics, Stanford University, Stanford, CA, [2]Chemistry and Chemical Biology, Harvard University, Cambridge, MA, [3]Biochemistry, University of Wisconsin-Madison, Madison, WI, [4]Applied Physics, Stanford University, Stanford, CA, [5]Biology, Stanford University, Stanford, CA

In prokaryotes, transcription termination is generally triggered by specific DNA sequence elements that encode an RNA transcript consisting of a GC-rich hairpin followed by a 9 nt U-rich tract. Although these sequence elements have been identified and characterized biochemically, questions still remain about the physical mechanism by which they lead to destabilization of the transcription elongation complex (TEC). The forward translocation model proposes that formation of the terminator hairpin pulls on the 5' end of the RNA, driving the RNAP molecule downstream along the DNA template without transcript elongation and shortening the RNA:DNA hybrid by up to several base pairs. The U-rich tract, which is also necessary for termination, is thought to facilitate transcript release by forming a comparatively weak RNA:DNA hybrid in the RNAP active-site cleft. To investigate the mechanistic role of these sequence elements, we used single-molecule optical trapping techniques to exert controlled forces on either the DNA template or the RNA transcript of individual *E. coli* TECs as these transcribed three terminator sequences (*his*, t500, and λtR2). Assisting or hindering force exerted through the DNA did not affect termination efficiency (TE) in WT terminators, indicating that the commitment step in termination does not likely involve forward translocation of RNAP along the DNA. However force-dependent transcript-release kinetics of the t500 terminator and the TE force-dependence of a t500 mutant suggest a forward translocation mechanism for this particular terminator. Tension along the RNA at terminator U-tracts (without hairpins) caused force-dependent transcript release, likely through shearing of the RNA:DNA hybrid. We deduce that formation of the last terminator hairpin bases supplies energy to destabilize the hybrid, and propose a quantitative energetic model that predicts the force-dependent TE of three WT terminators and the bulk TE of mutant terminators. Larson MH, Greenleaf WJ, Landick R, and Block SM. Applied force reveals mechanistic and energetic details of transcription termination. Cell (2008) 132: 971-982.

2762/B474

Long-range Single DNA Molecule Haplotyping of Human Clinical Radiation Sensitivity-associated Genes (PTTG1 and CD44).

Y. Michikawa; Research Center for Charged Particle Therapy, National Institute of Radiological Sciences, Chiba, Japan

OBJECTIVES: Haploid chromosomes distinguished by multiple SNP markers in human PTTG1 (Chromosome 5) and CD44 (Chromosome 11) genes have been reported to associate with the severity of adverse effects induced by radiation therapy of breast cancer patients. It is of interest to understand biological consequences of the heterozygotic state by functionally distinct, homologous haploid chromosomes within a nucleus of cells in the patients. To this end, diplotype of these chromosomes must be precisely determined to evaluate their clinical outcomes. METHODS: In this study, a novel methodology of long-range (over 20 kb) haplotype determination involving sensitive amplification of single DNA molecules within agarose gels has been developed. Limited dilution of DNA molecules was performed in heated alkaline agarose solution, avoiding extensive shearing and aggregation during dilution process. Aliquoting the gel solution provided physical separation of DNA molecules derived from homologous chromosomes. The solidified agarose gel pieces were then treated by exogenously supplied Phi29 DNA polymerase and random hexamer oligonucleotides, yielding up to 120,000-fold multiple-displacement amplification of the gel-embedded DNA molecules. The amplified materials were recovered in solution as PCR-ready form by simple heating, making them conveniently available for further multiple locus genotyping. RESULTS and CONCLUSIONS: Feasibility of this methodology for isolating long-range single DNA molecules spanning the selected SNP markers (PTTG1: 20 kb, CD44: 80 kb) was further confirmed using DNAs derived from patients with heterozygotic state. Thus, the haploid chromosomes distinguished by the SNP markers of the above clinical radiation sensitivity-associated genes can now be successfully determined in the individual breast cancer patients.

Nuclear Import and Export Signals (2763 – 2783)

2763/B477

Dissecting the Mechanisms of Stress-Induced Nuclear Accumulation of Nuclear Transport Factors with New Quantitative Imaging Techniques.

M. Kodiha, P. Banski, D. Ho-Wo-Cheong, H. Mahboubi, L. Johns, N. Crampton, U. Stochaj; McGill University, Montreal, QC, Canada

Nuclear transport of proteins is essential to all aspects of eukaryotic cell physiology; it requires the cooperation of several soluble factors. As such, classical nuclear import depends on the dimeric carrier importin-α/β1, and CAS, a member of the importin-β family, which exports importin-α to the cytoplasm. The physiological state of eukaryotic cells controls nuclear trafficking of many cargos, and stress triggers the inhibition of classical protein import. This is characterized by the redistribution of several transport factors, including importin-α and CAS which accumulate in nuclei of heat-shocked cells. The mechanisms underlying this relocation are poorly understood. Here, we designed *In Vivo* and *in vitro* studies in HeLa cells to identify the mechanisms that underlie the stress-induced nuclear concentration of importin-α and CAS. To accurately determine subtle changes in the subcellular distribution of transport factors we developed quantitative immunolocalization techniques that combine confocal microscopy with computer based image analysis. These new methods measure reliably fluorescence intensities in different cellular compartments such as the nucleus, cytoplasm or nuclear envelope. Our research defines at the molecular level the reactions that lead to the heat-induced nuclear accumulation of importin-α and CAS, and we demonstrate that different mechanisms contribute to their stress-induced nuclear accumulation. For importin-α, heat upregulates the docking at the nuclear envelope, drastically increases retention in the nucleoplasm and compromises exit to the cytoplasm. For CAS, heat stress significantly stimulates the translocation into the nuclear interior, whereas nuclear exit and retention are less affected. Taken together, our results support the idea that heat shock regulates importin-α and CAS nuclear accumulation on multiple levels. The combination of different stress-induced changes leads to the nuclear concentration of both transport factors in heat-stressed cells, ultimately contributing to the inhibition of classical nuclear import.

2764/B478

Calmodulin-Dependent Nuclear Import of the Testis-Determining Factor SRY.

G. Kaur, I. Poon, A. Delluc-Clavieres, D. J. Glover, K. M. Wagstaff, D. A. Jans; Biochemistry and Molecular Biology, Monash University, Clayton, VIC, Australia

SRY (Sex determining Region on the Y chromosome) is a key mediator of testis development, with a number of mutations that result in human XY sex reversal mapping to one of SRY's independently acting nuclear localisation signals (NLSs) that flank its DNA binding HMG (high mobility group) box. The C-terminal NLS (C-NLS) targets SRY to the nucleus through the conventional nuclear import receptor importin-β1 (Imp-β1), but no importin has been shown to bind the N-terminal NLS (N-NLS), although it is known to interact with the Ca^{2+}-binding protein calmodulin (CaM). In this study, we examine missense mutations in the SRY N-NLS from XY sex-reversed females for effects on nuclear import and ability to interact with CaM/Impβ1 for the first time. The mutations were all found to result in reduced nuclear localisation in transfected cells compared to wild type. The CaM antagonist, calmidazolium chloride (CDZ), was found to significantly reduce SRY nuclear accumulation indicating a CaM-dependent mode of SRY nuclear import. Intriguingly, N-NLS mutants were resistant to CDZ's effects, implying a loss of interaction with CaM, which was confirmed directly by *in vitro* binding experiments. Impaired CaM binding and the consequent nuclear localisation appeared to be the basis of sex-reversal in patients carrying the M64T and R76P mutations. CaM- and Imp-β1-dependent SRY nuclear import was able to be reconstituted *in vitro*, with reduced nuclear accumulation observed in the presence of antibodies to either CaM or Imp-β1. Intriguingly, *in vitro* binding assays indicated mutual exclusivity of binding of Imp-β1 and CaM to SRY. Our results strongly implicate a CaM-dependent nuclear import pathway for SRY mediated by the N-NLS that, together with the C-NLS, is essential to achieve threshold levels of SRY in the nucleus for male sex determination during foetal development.

2765/B479

mRNA Export Is Controlled by Coupling between FG-Nup Binding and RNP Remodeling at the Cytoplasmic Nuclear Pore Complex Face.

L. Terry, S. Wente; Department of Cell & Developmental Biology, Vanderbilt University Medical Center, Nashville, TN

The nuclear pore complex (NPC) coordinates selective and efficient trafficking of macromolecules between the nucleus and cytoplasm. During nucleocytoplasmic transport, a signal-bearing macromolecule is bound by a transport receptor. These transport receptors bridge interactions between the transport cargo and a subset of NPC proteins, the FG-Nups. Transient binding to the phenylalanine-glycine (FG) repeat domains is essential for facilitated translocation. To analyze the transport mechanism, we systematically generated combinatorial deletions of multiple FG domains from Saccharomyces cerevisiae and examined the transport capacity of these mutant minimal NPC strains. The FG domains required for mRNA export were distinct from those required for protein import. We speculate that the NPC regulates traffic flow by coordinating multiple FG translocation pathways, each specific to a transport receptor and its cargoes. We have now further employed these NPC mutant strains to identify factors required for terminal steps in mRNA export. The directional release of protein cargoes from transport receptors following movement through the NPC has been well characterized, but the terminal release steps in mRNA export are not fully delineated. Interestingly, the mRNA export defect in a mutant lacking FG domains from both the nucleoplasmic face and Nup57 was significantly rescued by the deletion of cytoplasmic face FG domains. This suppression was specifically due to deletion of the Nup42 FG domain. We and others recently defined roles for the DEAD-box protein Dbp5 and the mRNA export factor Gle1 at the NPC cytoplasmic face. Gle1 activates Dbp5 to trigger changes in the protein-bound mRNA composition and possibly release of the transport receptor Mex67/Mtr2. Of note the Nup binding sites for Dbp5 and Gle1 are juxtaposed to FG domains in cytoplasmic Nups. Using double mutant analysis and functional assays, we found mRNA export was specifically affected by the proximity of an FG-Nup binding site for the mRNA export receptor Mex67/Mtr2 to the NPC binding sites for the mRNA export factors Gle1 and Dbp5. We propose a model wherein juxtaposed NPC binding sites for Mex67/Mtr2, Gle1, and Dbp5 regulate the release of mRNA from the NPC.

2766/B480

Membrane Proteins Employ Multiple Mechanisms to Translocate from the Er to the Inner Nuclear Membrane.

N. Zuleger, D. A. Kelly, E. Schirmer; Wellcome Trust Centre for Cell Biology, Edinburgh, United Kingdom

Previous fluorescence recovery after photobleaching (FRAP) studies on well characterized nuclear envelope transmembrane proteins (NETs) Emerin, LAP2, MAN1 and LBR found that their velocities were very similar, possibly due to similar characteristics of their nucleoplasmic domains. Here we directly compared 15 NETs for their mobilities in the NE and found that NET velocities actually vary enormously. Testing a subset of NETs with photoactivatable constructs revealed that diffusion in the nuclear envelope is actually much slower even for the well-characterized NETs than previously reported whereas photoactivation in the ER yields similar kinetics as measured by FRAP. This suggests that much of what was previously measured is movement from the ER to the NE rather than movement within the NE. Targeting of NETs from the ER to the nuclear envelope is thought to occur by lateral diffusion in the membrane and retention at the nuclear envelope through binding lamins or chromatin. Reconsidering the FRAP studies as predominantly measuring movement from the ER to the NE, the wide range of velocities suggests that there are multiple mechanisms for transport to the inner nuclear membrane. Slower NETs tended to have a predicted NLS and more nucleoplasmic mass while the faster NETs lack a NLS and tend to have minimal nucleoplasmic mass. Interestingly, nearly all of the hundreds of transmembrane proteins identified by NE proteomics have nucleoplasmic masses smaller than the predicted diffusion limit for the peripheral channels of the NPC, further supporting a core mechanism involving lateral diffusion. The NETs were also tested for an energy requirement in translocation by ATP-depletion and only a subset were affected. We are currently testing a role for the Ran GTPase that is involved in classical nuclear transport pathways. Thus different NETs were directly compared in the same experimental system and it appears that different subsets of NETs are translocated via distinct mechanisms: the mechanism chosen for the transport seems to depend of the unique NET characteristics.

2767/B481

Structural Basis for the Function of Sus1, Sac3 and Cdc31 in Integrating mRNA Nuclear Processing and Export.

D. Jani[1], S. Lutz[2], N. Marshall[1], E. Hurt[2], M. Stewart[1]; [1]MRC Laboratory of Molecular Biology, Cambridge, United Kingdom, [2]BZH, University of Heidelberg, Heidelberg, Germany

There is an emerging consensus that transcription, processing and nuclear export of mRNA are linked. In yeast, Sus1 facilitates this coupling by linking the SAGA acetyltransferase complex to the Sac3:Cdc31:Thp1 (TREX-2) complex that is itself linked to the nuclear face of nuclear pores through an interaction between Sac3 and the nucleoporin, Nup1. This linkage is important in facilitating gene gating - the association of actively-transcribing genes with nuclear pores. We describe here the crystal structure of the macromolecular complex formed between Cdc31, Sus1 and Sac3 residues 723-805 that is crucial for integrating the expression pathway of a broad range of genes in Saccharomyces cerevisiae. This region of Sac3 forms a single long, continuous, gently undulating alpha-helix, whereas Sus1 is constructed from five alpha-helices arranged to form an antiparallel articulated hairpin that wraps tightly around the Sac3 alpha-helix. Highly conserved hinges located between the Sus1 helices provide crucial flexible joints that enable the molecule to wrap around Sac3 in a manner analogous to the way in which the fingers of a hand grasp a rod. The way in which Sus1 wraps around the Sac3 helix generates a large hydrophobic interaction interface between these two proteins while still leaving an extensive surface on Sus1 free to interact with other components of the gene expression machinery, such as the SAGA complex and the nuclear pore. The four EF-hands of Cdc31 take up an "open" calmodulin-like conformation and the molecule wraps around residues 785-805 of the Sac3 helix in a manner similar to that seen when it binds to the spindle pole body protein, Sfi1. We used the structural information we had obtained of the interaction interfaces between the components of the Sac3:Cdc31:Sus1 complex to engineer Sac3 mutants in which the binding to either Sus1 or Cdc31 was ablated while binding was retained to the other partner. These mutants were then used to probe the function of these interactions and showed defects in gene expression in budding yeast as well as providing powerful insight into the ways in which Sus1, Sac3 and Cdc31 contribute to the process.

2768/B482

Regulation of Nuclear Import of the Aristaless-Related Homeobox Protein.

T. Tao[1], W. Lin[1], W. Ye[1], X. Meng[1], L. Cai[1], Y. Yu[2], J. Golden[3], A. Tartakoff[4]; [1]Xiamen University, Xiamen, China, [2]University of Houston & M.D. Anderson Cancer Center, Houston, Houston, TX, [3]Children's Hospital of Philadelphia, Philadelphia, PA, [4]Case Western Reserve University, Cleveland, OH

Nuclear import of proteins with nuclear localization signals (NLSs) is mediated by shuttling carriers, the importins. Some cargoes display more than a single NLS, and among these are homeodomain proteins such as Arx, which is critical for development of multiple tissues. Arx has two functional NLSs. The present studies show that several pathways can import Arx via its NLS2, which is within its DNA-binding homeodomain. Using an in vitro nuclear import assay we show that import of Arx via NLS2 can be mediated by importin β1, importin 9 or importin 13 with binding being strongest to importin β1. All binding is sensitive to RanGTP. Experiments based on precise domain deletions indicate that NLS2 binds imp9 and imp13 and includes both an importin binding subdomain and a regulatory subdomain with arginine 382 being critical for function of NLS2. Interestingly, although importin α1 does not interact with intact Arx, it binds upon removal of sequences in or adjacent to the homeodomain. Moreover, importin α1 can mediate import of an NLS2-containing fragment in vitro, regardless of whether arginine 382 is mutated. We propose a novel and possibly quite general mechanism for regulation of nuclear import of DNA-binding proteins which are critical for development.

2769/B483

Involvement of a Novel NoDS^{h+} Sequence in the Targeting of Proteins to the Nucleolus.

I. Robert, M. Khacho, J. Dias, S. Lee; Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada

A consequence of anaerobic metabolism is the acidification of the extracellular milieu which is associated with certain physiological and pathological conditions such as muscle stress, ischemia and tumorigenesis. Extracellular acidosis induces relocalization and static detention of the VHL tumor suppressor protein in the nucleolus until neutral pH conditions are reinstated. Nucleolar VHL loses its function of targeting the hypoxia-inducible factor alpha (HIFα) for proteasomal degradation resulting in transcriptional activation of hypoxia-inducible genes.

Moreover, pH-dependent sequestration of VHL in nucleoli decreases rRNA synthesis, the most energy consuming cellular process. This reduces the cellular energy demand in conditions of limited energy supply resulting in maintenance of energy equilibrium during anaerobic metabolism. Nucleolar detention of VHL is directed by a specific motif named $NoDS^{H+}$ (nucleolar detention signal regulated by H^+) which is composed of an arginine rich domain and hydrophobic domains. Through a detailed database search, 18 proteins involved in basic cellular processes were found to harbour a $NoDS^{H+}$ code. Mutational analysis of the $NoDS^{H+}$ confirms the importance of the arginine domain for its activity as substitution or mutation of key residues considerably reduces or abrogates nucleolar sequestration. To further understand the mechanism used by cells to regulate nucleolar detention of proteins, we analyzed by mass spectrometry the $NoDS^{H+}$. Data obtained from mass spectrometry analysis suggest differential posttranslational modifications within the arginine domain between neutral and acidic conditions. Taken together, these results demonstrate the stringency of the $NoDS^{H+}$ code and suggest that posttranslational modifications might regulate its activity in detaining $NoDS^{H+}$-containing proteins in the nucleolus upon H^+ accumulation.

2770/B484

Cellular Adaptation to Anaerobic Metabolism through Nucleolar Sequestration of $NoDS^{h+}$ Proteins.

J. Dias, M. Khacho, I. Robert, S. Lee; Cellular and Molecular Medicine, University of Ottawa, Ottawa, ON, Canada

Anaerobic metabolism as a consequence of low oxygen tension (hypoxia) is observed in various physiological and pathological conditions including strenuous exercise, ischemic stroke, ischemic heart disease, anemia and tumorigenesis. Though hypoxic cells maximize energy production through increasing their glycolytic rate, this far from compensates for loss of mitochondrial energy production. Therefore it would be reasonable to envision that in these conditions cells also decrease demand in order to maintain energy equilibrium, a concept which has been given little focus. Through the study of adaptive mechanisms to anaerobic metabolism, it was recently shown that an increase in the extracellular $[H^+]$ causes the relocalization and sequestration of the von Hippel-Lindau (VHL) tumor suppressor to the nucleolus. This results in an indirect increase in energy production through HIF transcription factor stabilization and a decrease in energy demand through silencing of ribosomal biogenesis. Mutagenesis of VHL revealed a pH-dependent nucleolar targeting sequence, $NoDS^{H+}$. A bioinformatic search of this sequence identified proteins involved in major metabolic activities including; POLD1, the catalytic subunit of DNA polymerase delta, TAF1, subunit of the general transcription factor TFIID, and UAP56, an mRNA splicing and export factor. Here we demonstrate that in response to acidic conditions, these proteins become targeted to the nucleolus. Functional studies show that nucleolar POLD1 correlates with a significant decrease in DNA replication which can be rescued through exogenous expression of a reconstituted $NoDS^{H+}$ of POLD1. Disruption of sequestration interferes with the protective effects of acidosis in hypoxia resulting in increased cell death. These results support a model in which cells disrupt major metabolic pathways through nucleolar sequestration in order to maintain energy equilibrium in anaerobic metabolism.

2771/B485

Exposure of Nuclear Localization Sequences Regulates the Nuclear Trafficking of the Influenza a Virus.

W. W. Wu, N. Pante; Department of Zoology, University of British Columbia, Vancouver, BC, Canada

The influenza A viral RNA genome, complexed with oligomeric viral nucleoprotein (NP), must enter the nucleus to allow for viral transcription and translation of new viral proteins. Subsequently, nuclear entry of newly-synthesized NP allows for assembly and nuclear export of newly-synthesized viral RNA-NP complexes (vRNPs). The viral mechanism by which nuclear import is mediated is thus a key part of the influenza A life cycle. We previously found that nuclear import of purified vRNPs is mediated by two nuclear localization signals (NLS1 and NLS2, at the N-terminus and middle of NP, respectively), with NLS1 being the stronger mediator due to its higher surface exposure. However, it is unknown whether these NLSs display a similar degree of exposure on newly-synthesized NP and on newly-assembled vRNPs, and whether they are differentially masked during the influenza A virus life cycle. The objective of this study was therefore to address these questions. To do so, we examined the exposure of these NLSs on newly-synthesized vRNP and NP in influenza-infected and NP-transfected cells by immuno-fluorescence microscopy with antibodies against these NLSs. Similar to purified vRNPs, our results showed that NLS1 was generally more highly exposed than NLS2. Interestingly, NLS2 was only exposed in the nucleus, while NLS1 was exposed in the cytoplasm and nucleus. However, NLS1 and NLS2 were hidden in nuclear-exported vRNPs, likely as a mechanism to prevent vRNP nuclear re-entry. These NLSs also had differential nucleolar exposure. Together, these results indicate that NLS1 and NLS2 are exposed in cells after influenza infection or NP transfection to mediate the nuclear import of incoming vRNPs and newly-synthesized NP, and these NLSs become hidden on nuclear-exported vRNPs. We therefore propose that the selective exposure of vRNP NLSs constitutes a critical mechanism to regulate the directionality of its nuclear trafficking during the influenza A viral life cycle.

2772/B486

Individual Binding Pockets of Importin β; for FG-Nucleoporins Have Different Binding Characteristics and Different Sensitivities to RanGTP.

S. Otsuka, K. Takeyasu, S. Yoshimura; Graduate School of Biostudies, Kyoto University, Kyoto, Japan

Background: Importin β; mediates protein transport across the nuclear envelope (NE) through the nuclear pore complex (NPC) by interacting with components of the NPC, called nucleoporins, and a small G protein, Ran. Although there is accumulated knowledge on the specific interaction between importin β; and the Phe-Gly (FG) motif of the nucleoporins as well as the effect of RanGTP on this interaction, the molecular mechanism by which importin β; shuttles across the nuclear envelope through the NPC is unknown. *Results:* We focused on four binding pockets of importin β; for the FG motifs and characterized the interaction utilizing a single-molecule force-measurement technique with atomic force microscopy. A purified FG-Nup was immobilized on a glass substrate and GST-fused importin β; was non-covalently attached to a glutathione-coupled AFM cantilever and then the strength of the importin β;-FG-Nup-interaction was evaluated at a single-

molecule level. The results from a series of importin β; mutants containing amino acid substitutions within the FG-binding pockets demonstrate that the individual FG-binding pockets have different affinities to FG-Nups (those of Nup62 and Nup153) and different sensitivities to RanGTP; the binding of RanGTP to the amino-terminal domain of importin β; induces the conformational change of the entire molecule and reduces the affinity of some of the pockets, but other pockets still retain the affinity. The effect of RanGTP on the interaction between importin β; and the nucleoplasmic side of the NPC was also investigated. A NE dissected from a *Xenopus* oocyte was spread onto a glass surface and then rupture force was measured in the presence and absence of RanGTP. The measurement demonstrated that the affinity between importin β; and the nucleoplasmic face of the NPC was reduced by RanGTP, implying that some of the pockets, but not all, are inactivated by RanGTP. *Conclusion:* This line of evidence suggests that these interactions are involved in the directional transport of importin β; from the cytoplasm to the nucleoplasm.

2773/B487

Specific Mutations in LTV1 Affect 40S Ribosome Subunit Export and Cytoplasmic Maturation.

C. Fassio[1], B. Schofield[1], R. Seiser[2], A. Johnson[3], D. Lycan[1]; [1]Biochemistry/Molecular Biology, Lewis and Clark College, Portland, OR, [2]Department of Biological, Chemical and Physical Sciences, Roosevelt University, Chicago, IL, [3]Molecular Genetics and Microbiology, University of Texas at Austin, Austin, TX

The S. Cerevisiae protein Ltv1 is a conserved nonessential protein required for efficient 40S ribosome biogenesis. It is a component of late pre-40S subunits and has been proposed to function in nuclear export, perhaps recruiting the karyopherin Crm1. To test this hypothesis, we mutated a putative NES in LTV1. We predicted, by analogy with NES- mutations in the 60S subunit export adapter, that expression of the mutant protein would be dominant lethal and lead to the accumulation of 40S subunits in the nucleus. We find that over-expression of the mutant protein is indeed dominant negative for growth in a dosage-dependent manner, in both wild-type and Δltv1 cells. However, mutant ltv1-GFP is not nuclear-localized. To further explore this unexpected result, we examined polysome profiles and find that cells over-expressing mutant ltv1 exhibit reduced 40S levels and increased levels of free 60S subunits, indicative of defects in 40S assembly or maturation. The mutant protein is efficiently incorporated into pre-40S subunits, as western blots of polysome gradients show most of the mutant protein in the 40S peak and not as free protein. Over-expression of mutant ltv1 is associated with significant nuclear retention of the 40S proteins RpS2-GFP and RpS3-GFP, indicative of an export defect. However, in addition, in-situ hybridization using a fluorescent probe directed against an rRNA internal transcribed spacer sequence (5'-ITS1) suggest that cells over-expressing mutant ltv1 are also deficient in the cytoplasmic cleavage of 20S to 18S rRNA, a maturation step required before 40S subunits are functional for translation. One interpretation of these results is that the dominant negative effect of mutant Ltv1 is due to interruption of pre-40S subunit maturation in the cytoplasm, which in turn negatively feeds back on nuclear export.

2774/B488

Reengineering Ribosome Export.

K. Lo, A. Johnson; Molecular Genetics and Microbiology and the Institute for Cell and Molecular Biology, UT Austin, Austin, TX

The large (60S) ribosomal subunit is one of the bulkiest cargoes to be transported through the nuclear pore complex. It is exported out of the nucleus by the combined effort of three different export receptors: Crm1, recruited through the adapter protein Nmd3; the mRNA export receptor Mex67/Mtr2, and the noncanonical export receptor Arx1. Of these, only Nmd3/Crm1 appears to have retained this 60S export function in higher eukaryotes. However, efficient export of large cargoes requires multiple receptors, suggesting that higher eukaryotes have evolved other proteins for export. We decided to determine if there is a requirement for specific export receptors for the large ribosomal subunit. We did this my making direct protein fusions between Nmd3, deleted of its nuclear export signal (NES), and various export receptors. Surprisingly, the fusion of Mex67 to truncated Nmd3 lacking its Crm1-dependent NES restored export function to Nmd3. We then tested fusions of other export receptors that are not involved in ribosome export, including Los1, the tRNA export receptor in yeast. Fusions of Mtr2, Cse1, Los1, or Msn5 to the Nmd3 NES-deficient mutant all suppressed the lethality of a conditional nmd3 mutant. Furthermore, these chimeric proteins supported 60S export. Thus, there does not appear to be a requirement for a specific export receptor for the large subunit. Because these chimeric proteins bind directly to the ribosome through their Nmd3 moieties, they are not released by GTP hydrolysis on Ran, raising the question of how they provide directioality for transport. We show that the fusion proteins are dependent on the GTPase activity of Lsg1, the cytoplasmic release factor for Nmd3. Finally, we asked if export could be driven by adding an NES to the ribosome in cis, Indeed, fusing the NES of Nmd3 to the large subunit ribosomal protein, Rpl3, was sufficient to drive export of the large subunit in cells lacking Arx1 and partially impaired for Nmd3 function. These results imply remarkable flexibility in the nature of the export receptor and export signal for the large ribosomal subunit and may help explain how different export receptors could have evolved in different eukaryotic lineages.

2775/B489

Nuclear Import of Parvovirus Involves Caspase-Mediated Nuclear Envelope Disruption.

S. Cohen, N. Pante; Zoology, University of British Columbia, Vancouver, BC, Canada

Parvoviruses are small, non-enveloped single stranded DNA viruses. In order to replicate successfully parvoviruses must enter the nucleus of their host cells. However, the nuclear envelope (NE) acts as a barrier between the cytoplasm and the nucleus, and nuclear import is tightly regulated by the nuclear pore complexes (NPCs). Although little is known about the mechanisms underlying parvovirus' nuclear import, it has been assumed that parvoviruses enter the nucleus through the NPC. To study this problem we microinjected the parvovirus Minute virus of mice (MVM) into the cytoplasm of Xenopus oocytes and used electron microscopy to visualize its nuclear import. Unlike oocytes microinjected with other import substrates that have shown the substrate crossing the NPC, MVM was not seen in transit through NPCs. Instead, we found that injection of MVM caused disruption of the nuclear membranes. Often it appeared that virus particles were associated

with the NE, or between the outer and inner nuclear membranes in close proximity to these disruptions. Similar results were found in mouse fibroblast cells infected with MVM, even at early time points after infection. Our data indicate that MVM induces small disruptions of the nuclear membranes and enters the nucleus through the resulting breaks. This is a novel type of mechanism that has never been described for the nuclear import of any virus or protein. Our hypothesis is that MVM hijacks cellular mechanisms for nuclear envelope breakdown (NEBD), such as those used during mitosis and apoptosis. To test this hypothesis we have used a semi-permeabilized cell assay for NE disruption by MVM in the presence of various inhibitors of enzymes known to be involved in NEBD during apoptosis. We found that NE disruption by MVM was inhibited by several inhibitors of caspases. We propose that parvoviral nuclear import involves the hijacking of cellular factors to initiate a program for NEBD similar to that used by the cell during apoptosis.

2776/B490

Biochemical Analysis of the Interaction of the Human Proto-Oncogene Nup214/CAN with the DEAD-box Helicase Ddx19.

J. Napetschnig[1], S. Kassube[1], R. Wong[1,2], A. Hoelz[1], G. Blobel[1]; [1]The Rockefeller University, New York, NY, [2]Cancer Research Institute, Kanazawa University, Kanazawa, Japan

A key step of mRNA export is the release of mRNA from the cytoplasmic side of the nuclear pore complex (NPC) into the cytoplasm. This process is facilitated by export factors associated with the mRNA and their interaction with the NPC. One such complex includes the DEAD-box helicase Ddx19 and the proto-oncogene Nup214/CAN. Recruitment of Ddx19 to the cytoplasmic side of the NPC is dependent on the N-terminal domain of Nup214/CAN and is essential for mRNA export in yeast. We previously solved the structure of the N-terminal domain of Nup214/CAN revealing a seven-bladed β-propeller and identified several putative protein interaction sites. Here we present a biochemical and in vivo analysis of the interaction between Nup214/CAN and Ddx19. We identify the protein binding sites and demonstrate that the interaction between Ddx19 and Nup214/CAN is electrostatic. We reveal that Ddx19 has a composite binding site on Nup214/CAN and show that residues 343 to 351 of the conserved 6D7A loop of Nup214/CAN are necessary but not sufficient for complex formation. Furthermore, we show that predominantly domain 1 of Ddx19 is involved in binding to Nup214/CAN and identify a conserved residue of Ddx19 that is essential for complex formation in vitro. Finally, we demonstrate that the positive charge of this residue is necessary for binding of Ddx19 to the nuclear envelope in vivo.

2777/B491

mDia2 Shuttles between the Nucleus and the Cytoplasm through the Importin-β- and CRM1-Medaited Nuclear Transport Mechanism.

T. Miki[1], K. Okawa[2], T. Sekimoto[3], Y. Yoneda[3], S. Watanabe[1], T. Ishizaki[1], S. Narumiya[1]; [1]Pharmacology, Kyoto University Faculty of Medicine, Kyoto, Japan, [2]Frontier Technology Center, Graduate School of Medicine, Kyoto University, Kyoto, Japan, [3]Biochemistry, Graduate School of Medicine, Osaka University, Suita, Japan

Mammalian homolog of *Drosophila* diaphanous (mDia) consisting of three isoforms, mDia1, mDia2 and mDia3, is an effector of Rho GTPases that catalyzes actin nucleation and polymerization. While the mDia actions on actin dynamics in the cytoplasm have been well studied, whether mDia accumulates and functions in the nucleus remains largely unknown. Given the presence of actin and acitn-associated proteins in the nucleus, we have examined nuclear localization of mDia isoforms. We expressed each of mDia isoforms as a GFP-fusion protein in HeLa cells and examined their localization. We found; all the mDia isoforms are localized predominantly in the cytoplasm of cells under steady-state culture conditions. However, upon treatment with leptomycin B (LMB), an inhibitor of CRM1-dependent nuclear export, mDia2 and not mDia1 or mDia3, accumulated in the nucleus. The LMB-induced nuclear accumulation of mDia2 was confirmed for endogenous protein by using antibody specific to mDia2. Studies using GFP-fusion of various truncation mDia2 mutants and point mutants of some of these constructs identified a functional nuclear localization signal (NLS) in the N-terminus of mDia2, and at least one functional nuclear export signal (NES) in the C-terminus. Exogenously expressed mDia2 fragment containing the NLS co-precipitated with Importin-β from the cell lysates, and depletion of Importin-β with RNA interference suppressed the LMB-induced nuclear localization of endogenous mDia2. These results suggest that mDia2 continuously shuttles between the nucleus and the cytoplasm using specific nuclear transport machinery composing of Importin-β and CRM1.

2778/B492

KPNA4-16: Characterization of a Sorting Protein That Directs Integral Membrane Proteins to the Inner Nuclear Membrane.

X. Wu[1], S. Braunagel[2], M. D. Summers[1,2]; [1]Entomology, Texas A&M University, College Station, TX, [2]AgriLife Research, Texas A&M System, College Station, TX

KPNA4-16, a 16-kDa polypeptide encoded from KPNA4 is a mammalian isoform of *Spodoptera frugiperda* importin-α-16, which is a translocon-associated protein involved in sorting membrane proteins to the nuclear envelope. To understand how KPNA4-16 facilitates the transport of integral membrane proteins to the inner nuclear membrane (INM), we identified protein-interaction partners of KPNA4-16. Two approaches were undertaken to discover partners of KPNA4-16; a GST pull-down assay and direct binding assays. For the GST pull down assay, GST-KPNA4-16 was bound to beads and overlaid with Hela cell cytoplasmic and nuclear fractions. The bound protein complexes were separated by SDS-PAGE and proteins identified using mass spectrometry. The identity of proteins binding to KPNA4-16 revealed Dis3, CAS and others. Subsequently, direct protein-protein binding assay were performed using human genes expressed as GST fusions and purified by TEV digestion. The direct binding assays confirmed the GST-pull down assay results and identified additional interacting partners, such as Nup50. In parallel, a yeast isoform of KPNA4-16 was tagged with protein A-ZZ domain and expressed in yeast. Yeast cells carrying the Kap60-44-ZZ plasmid were bound to IgG beads and the resultant protein complexes separated using SDS-PAGE and analyzed using mass spectrometry. Use of a recombinant baculovirus determined that importin-α-16 remains with the INM-directed protein as it passes the pore membrane and is trafficked to the INM. The identification of putative protein partners of KPNA4-16 and knowledge of how long the protein complex stays together allows the development of a model of the molecular pathway of trafficking of integral membrane proteins to the INM.

2779/B493

Hampin/msl1 Regulates Nuclear Transport of Putative Tumor Suppressor TTC4.

N. Pestov, R. Dmitriev, I. Okkelman, M. Shakhparonov; Shemyakin-Ovchinnikov Instituite of Bioorganic Chemistry, Moscow, Russian Federation

Hampin/msl1 is a well known partner of acetyltransferase MYST1 that is important for chromatin remodeling through histone H4 K16 acetylation. Recently, hampin was shown to interact with several other proteins including TTC4, a tetratricopeptide repeat protein. TTC4 is a little studied, but very interesting protein because it is involved in regulation of DNA replication and, putatively, in oncogenesis. In this work, we aimed at elucidating the functional consequences of the interaction between hampin and TTC4 in vivo. We found that endogenous TTC4 shuttles between the nucleus and cytoplasm in a cell cycle dependent manner. Rather unexpectedly, overexpression of TTC4 or TTC4-GFP in 3T3 fibroblasts shows that all excess TTC4 accumulates in the cytoplasm. When, however, hampin and TTC4 tagged with fluorescent proteins were co-expressed, a significant translocation of TTC4 to the nucleus was observed. Moreover, the proteins co-localized in certain nuclear foci. Both nuclear translocation of TTC4 and the focal co-localization was specific to hampin splice variants A, B and was completely absent with variants C, D, and E that lack the C-terminal PEHE domain. Interestingly, variant E showed even cytoplasmic localization in these co-expression experiments which is highly atypical for hampin that always demonstrates strict nuclear localization. Additionally, we found that hampin possesses two nuclear localization signals (NLS), one of them is present in all isoforms, and the second one is specific for the isoforms with PEHE domain. In conclusion, the nuclear localization of hampin is ensured by two NLS in different domains whereas TTC4 lacks its own NLS and its nucleocytoplasmic transport is controlled by protein-protein interactions. Nuclear translocation of TTC4 at least partly depends on hampin whereas its cytoplasmic accumulation may be caused by interactions with some unknown partner proteins.

2780/B494

Yeast Produce Multiple Isoforms of Kap60 and One of These Isoforms Affects Trafficking of Integral Membrane Proteins to the Nuclear Envelope.

D. Liu[1], K. Ryan[3], X. Wu[1], M. D. Summers[1,2], S. C. Braunagel[1,2]; [1]Entomology, Texas A&M University, College Station, TX, [2]AgriLife Research, Texas A&M System, College Station, TX, [3]Biology, Texas A&M University, College Station, TX

Earlier studies have shown that truncated isoforms of importin-α participate in directed trafficking of integral membrane proteins to the inner nuclear membrane. However, due to multiple genes and functional redundancy, it is difficult to study the unique function of these isoforms in a mammalian system. *Saccharomyces cerevisiae* however only encodes one importin-α-like protein (Kap60), as such it provides the ideal system to dissect the functional role of multiple isoforms of Kap60. To resolve if nested transcripts like those previously identified in *Spodoptera frugiperda* and Hela cells exist within the Kap60 gene in yeast, primer extension was performed. Five major internal transcripts were detected, encoding 3 truncated importin-α proteins (Kap60-44, Kap60-30 and Kap60-10). These isoforms are similar to those identified in insect and human cells. All of the isoforms lack the importin-β binding sequence and like those isoforms identified previously in other species, they share the same amino acid sequence, but each protein initiates at a different internal methionine. When all the internal methionines within Kap60 are mutated so that none of the isoforms can be translated, the classical nuclear localization pathway does not appear to be grossly affected (analyzed using cNLS-GFP), nor does the Ran gradient appear to be altered (analyzed using Nab2-GFP). However, cells lacking all the internal isoforms grow slower, and cannot grow under mild stress conditions. When the internal isoforms are deleted, the localization and enrichment of the INM protein heh2-GFP at the nuclear periphery does not occur, rather heh2-GFP is now detected throughout the ER. Rimming of heh2-GFP at the nuclear periphery can be restored when the mutated Kap60 and one internal isoform Kap60-44, is present. Membrane association of Kap60 and all the isoforms can be detected in cells. These data suggest that the observations made previously in insect and mammalian cells are also true for yeast. More than one form of Kap60 is present in the yeast cell and at least one of these affects efficient trafficking of proteins to the nuclear envelope.

2781/B495

Nuclear Pore Complex Assembly Is Linked to the Rates of Nuclear-Cytoplasmic Transport and Cell Cycle Progression.

D. Anderson[1,2], M. Hetzer[1]; [1]Molecular and Cell Biology Laboratory, Salk Institute, La Jolla, CA, [2]University of California San Diego, San Diego, CA

Transport between the nucleus and cytoplasm is regulated by nuclear pore complexes (NPCs), aqueous channels embedded in the nuclear envelope (NE). During interphase, NPCs are inserted into the NE, doubling in number before the next mitotic event. It is unclear if pore doubling, which is needed to keep NPC number constant for subsequent cell divisions, plays a role in the overall nucleo-cytoplasmic transport activity during interphase or in cell cycle progression. To address these questions we have developed a live-cell assay to measure the rate of nuclear transport. To test the importance of interphase pore insertion, NPC assembly was blocked by siRNA knockdown of Nup107, an essential component of the NPC. We found that the rate of nuclear import was dramatically decreased when pore number was reduced. Interestingly, this decrease in import resulted in a significant delay in the timing of interphase progression. Together these findings suggest that pore number limits the rate of nuclear-cytoplasmic transport and is a critical determinant for the rate of cell cycle progression. In addition to changes in NPC number through the cell cycle, the composition of pores change at the beginning of interphase when the nuclear compartment of metazoa is reestablished. This occurs by the recruitment of nuclear membrane and the stepwise reassembly of NPCs. For example, nup153, a pore component that is directly involved in nuclear import, has been shown to be recruit very early in nuclear assembly; where as Nup214, which participates in nuclear export, is not fully accumulated until after nuclei are formed. With the use of several fluorescent transport reporters, we found that NLS-mediated nuclear import is activated prior to NES-mediated export. Through siRNA knockdown, we determined a direct link between Nup153 and the asynchrony of import and export activation. From these data, we propose a model where NPCs begin nuclear import prior to their full assembly, however, nuclear export requires a completely formed pore. Together, these finding suggest that both NPC composition and number are important determinants of the dynamics of nuclear-cytoplasmic transport.

2782/B496

Fragile X Mental Retardation Protein FMRP Binds mRNA in the Nucleus and Is Exported in Part by Tap/NXF1.

M. Kim, M. Bellini, S. Ceman; University of Illinois-Urbana Champaign, Champaign, IL

The Fragile X mental retardation protein (FMRP) is an RNA binding protein that associates with a large collection of mRNAs and regulates their translation. Since FMRP was previously shown to be a nucleocytoplasmic shuttling protein, we examined the hypothesis that FMRP binds its cargo mRNAs in the nucleus. EGFP-FMRP expressed in Cos-7 cells was efficiently exported from the nucleus in the absence of its nuclear export sequence and even after a strong nuclear localization sequence was added, suggesting an efficient mechanism for export. We hypothesized that nuclear FMRP might exit the nucleus through its bound mRNAs. Using four independent silencing RNAs to reduce expression of the bulk mRNA exporter Tap/NXF1 we observed a significantly increased number of cells expressing EGFP-FMRP in the nucleus. Removal of FMRP's nuclear export sequence, in addition to Tap/NXF1 silencing, resulted in an even greater number of cells expressing FMRP in the nucleus, correlating with the presumed block in mRNA export. RNAse treatment released EGFP-FMRP from the nucleus and EGFP-FMRP interacted with Tap/NXF1 though an RNA intermediate. Further, isolation of complexes containing both EGFP-FMRP and Flag-Tap/NXF1revealed the presence of the FMR1 mRNA. To determine whether FMRP binds pre-mRNAs cotranscriptionally, we expressed HA-SV40 FMRP in amphibian oocytes and found it on the active transcription units of the lampbrush chromosomes. We also found endogenous Xenopus FMRP present on nascent transcripts. Collectively, our data provide the first lines of evidence that FMRP binds mRNA in the nucleus.

2783/B497

Two Distinct, Interacting Classes of Nuclear Envelope-Associated Coiled-Coil Proteins Are Required for the Tissue-Specific Nuclear Envelope Targeting of Arabidopsis Rangap.

J. Brkljacic, Q. Zhao, I. Meier; Department of Plant Cellular and Molecular Biology, Ohio State University, Columbus, OH

The Ran GTPase plays essential roles in multiple cellular processes, including nucleocytoplasmic transport, spindle formation and post-mitotic nuclear envelope (NE) re-assembly. The cytoplasmic Ran GTPase activating protein RanGAP is critical to establish a functional RanGTP/RanGDP gradient across the NE, and is associated with the outer surface of the NE in metazoan and higher plant cells. Arabidopsis (Arabidopsis thaliana) RanGAP association with the root tip NE requires a family of likely plant-specific nucleoporins combining coiled-coil and transmembrane domains (CC-TMD), WPP-domain interacting proteins (WIP). We have now identified, by tandem affinity purification coupled with mass spectrometry, a second family of CC-TMD proteins, structurally similar, yet clearly distinct from the WIP family, that is required for RanGAP NE-association in root-tip cells. A combination of loss-of-function mutant analysis and protein-interaction data indicates that at least one member of each NE-associated CC-TMD protein family is required for RanGAP targeting in root tip cells, while both families are dispensable in other plant tissues. This suggests an unanticipated complexity of RanGAP NE targeting in higher plant cells, contrasting both the single-nucleoporin anchor in metazoans and the lack of targeting in fungi and proposes an early evolutionary divergence of the underlying plant and animal mechanisms.

Chromatin and Chromosomes II (2784 – 2799)

2784/B498

Functional Comparison of Embryonic and Adult Linker Histones in Xenopus Egg Extracts.

B. Freedman, R. Heald; Molecular and Cell Biology, UC Berkeley, Berkeley, CA

Linker histone H1 is a fundamental player in chromatin fiber organization, but its roles in higher order chromosome structure and regulation are poorly understood. Histone H1 isoforms in adult animals are more positively charged than the oocyte/early embryonic variant H1oo, and possess several CDK1 phosphorylation sites, indicating cell cycle regulation. To probe the functional significance of these differences, we have compared H1oo to adult isoforms during sperm chromosome nuclear cycles in Xenopus egg extracts. During interphase, adult H1 isoforms cause chromatin hypercondensation at fivefold lower concentrations than H1oo. In mitosis, the situation is reversed, with H1oo binding and compacting chromosomes at significantly lower concentrations than adult histones H1A or H10. Intriguingly, adult H1 isoforms associate tightly with the nuclear transport proteins RanBP7 and importin beta, whereas H1oo does not. Phosphomutant analysis suggests that CDK1 phosphorylation of adult H1 isoforms enhances their ability to bind mitotic chromosomes. We hypothesize that H1oo has evolved as a linker histone uniquely suited to oocyte meiotic arrest and early embryonic divisions, whereas adult H1 isoforms function predominantly during interphase, and require more complex regulation during mitosis.

2785/B499

Drosophila Histone Variant H2AvD Is a Component of Centromere Nucleosome and Regulates Centromere Propagation.

W. Zhang[1,2], S. Colmenares[1,2], G. Karpen[1,2]; [1]Life Sciences Division, UC Berkeley & LBNL, Berkeley, CA, [2]Department of Molecular and Cellular Biology, University of California, Berkeley, Berkeley, CA

CENP-A (CID in Drosophila) is the centromere-specific histone H3 variant and a primary epigenetic regulator for centromere identity and kinetochore formation. Drosophila centromeres are comprised of alternative domains containing CID or canonical histone H3 nucleosomes. It has been previously indicated that the centromeric H3 nucleosomes have a characteristic pattern of histone modifications distinct from both euchromatin and heterochromatin. Here we identified H2Av, the histone H2A variant in Drosophila, as another important epigenetic regulator of centromeric chromatin. Using purified mononucleosomes, we demonstrated that H2Av is present in a subpopulation of CID nucleosomes as well as in alternative H3 nucleosomes at centromeres and flanking heterochromatin. We further showed evidence consistent with octameric native centromeric CID nucleosomes. By RNA interference (RNAi) experiments targeting H2Av in Drosophila S2 tissue culture cells, we demonstrated that H2Av is required for normal level of CID at centromeres. We studied the impact of H2Av on loading of newly synthesized CID using the SNAP system. We found that the centromeric level of newly synthesized CID significantly reduced in H2Av depleted cells. Our results provide evidence that the H2Av at centromeres are pivotal to normal centromere structure and functions.

2786/B500

Potential Role of the Histone Chaperone CAF-1 in Transcription.

J. Seol, H. Kim, E. Cho; College of Pharmacy, Sungkyunkwan University, Suwon, South Korea

Eukaryotic genome forms a chromatin structure with a nucleosome as a repeating unit. Its packaging state is regulated by chromatin remodeling factors, such as histone chaperones. The Saccharomyces cerevisiae H3/H4 histone chaperone, CAF-1 and Asf1 are involved in chromatin assembly/disassembly which is coupled to DNA replication while HIR and Asf1 are involved in a transcription coupled chromatin assembly/disassembly. Asf1 interacts with both CAF-1 and HIR, affecting both replication-coupled and transcription-coupled pathways. CAF-1 function is so far restricted to non-transcriptional process in the heterochromatin. Subunits of CAF-1 do not have significant genetic interaction with any elongation factors. Although Asf1 and CAF-1 play together in some aspects, the role of CAF-1 in euchromatin has not been elucidated. This study examined the role of the yeast H3/H4 histone chaperones, Asf1, HIR and CAF-1 in chromatin dynamics during transcription. Unexpectedly, CAF-1 was recruited to the actively transcribed region similar to HIR or Asf1. In addition, three histone chaperones genetically interacted with the Set2 dependent H3 K36 methylation, which suggests that histone methyltransferase Set2, Asf1, HIR, and CAF-1 function in a related pathway. Similar to histone chaperones, Set2 was required for the tolerance to excess histone H3 but not to excess H2A.

2787/B501

Lid2, a JmjC-Domain Histone Demethylase, Is a Key Regulator for Cross-Regulation of H3K4 and H3K9 Methylation of Both Euchromatin and Heterochromatin.

F. Li[1], M. Huarte[2], M. Vaughn[3], M. Zaratiegui[3], Y. Shi[2], R. Martienssen[3], W. Cande[1]; [1]Department of Molecular & Cell Biology, University of California Berkeley, Berkeley, CA, [2]Department of Pathology, Harvard Medical School, Boston, MA, [3]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Establishment of proper histone methylation pattern by various lysine residuals is essential for chromatin regulation. While H3K9 methylation is the hallmarks of heterochromatin, H3K4 methylation is usually associated with euchromatin. Little is known about the mechanism governing the coordination between H3K4 and H3K9 methylation. Here we have demonstrated that a S. Pombe JmjC-domain protein Lid2 is a tri-methyl H3K4 demethylase. We further revealed that Lid2 is a novel silencing regulator, interacting with the H3K9 methyltransferase, Clr4, through the Dos1/Clr8-Rik1 complex, which also functions in the RNA interference pathway. Disruption of the JmjC-domain alone results in severe heterochromatin defects and depletion of siRNA while overexpressing Lid2 enhances heterochromatin silencing. Thus, our biochemical and genetic studies established a direct physical and functional link between between H3K4 demethylation and H3K9 methylation suggesting a novel mechanism for coupling the two reactions in heterochromatin. Remarkably, our results indicated that Lid2 is also required for cross-regulation of H3K4 and H3K9 methylation in euchromatin and suggested euchromatin is regulated by dynamic interplay between H3K4 and H3K9 demethylases. The duality of Lid2 function between euchromatin and heterochromatin implicates a role in balancing the assembly and function of these two domains. These studies shed light on the mechanisms underlying the coordination of H3K4 and H3K9 methylation in both heterochromatin and euchromatin.

2788/B502

Cytoplasmic HDAC Profiling in *S. Cerevisiae.*

M. Wang[1], R. N. Collins[2]; [1]Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY, [2]Department of Molecular Medicine, Cornell University, Ithaca, NY

Reversible lysine acetylation has a well-defined role in nuclear events such as chromatin remodeling and has also been found on nuclear transport proteins, transcription factors, cell cycle components, coactivators and nuclear receptors. To date, much less is known about protein acetylation in the cytoplasm although several prominent examples have been identified such as α-tubulin and Hsp90. Recently, inhibitors of the mammalian, cytoplasmic and microtubule-associated deacetylase HDAC6 have received attention for their anti-proliferative effects that may be effective in treating certain forms of cancer. The identification of several cytoplasmic proteins that are the targets of acetylation together with the recognition that several deacetylases are cytoplasmically located suggest that acetylation may be playing a larger, unrecognized role in the cytoplasm. My project aims to study the biological roles of cytoplasmic histone deacetylases (HDACs) using S. cerevisiae as a model system. Through a fluorescence microscopy localization study spanning all genomic deacetylase proteins, I uncovered two HDAC proteins that are located outside the nuclear compartment and report on the biological significance and possible targets for these cytoplasmic HDAC proteins.

2789/B503

Epigenetic Regulation of *BDNF* Expression via RACK1.

D. He, M. Logrip, D. Ron; Ernest Gallo Research Center, University of California San Francisco, Emeryville, CA

We previously found that the activation of the cAMP/PKA pathway leads to the nuclear translocation of the scaffolding protein RACK1 resulting in the upregulation of brain-derived neurotrophic factor (*BDNF*) expression in hippocampal neurons (1). Here we set out to identify the molecular mechanism by which RACK1 regulates *BDNF* expression. Incubation of SH-SY5Y cells and primary hippocampal neurons with the adenylate cyclase activator forskolin resulted in an increase in the expression of *BDNF* exon III that was inhibited by siRACK1-recombinant adenovirus (AdV-siRACK1)-mediated downregulation of RACK1 level. Using a chromatin immunoprecipitation assay, we observed that forskolin treatment induces an interaction of RACK1 specifically with the chromatin complex at *BDNF* promoter III in SH-SY5Y cells. We then investigated the association of *BDNF* promoter III with MeCP2, the major methylated DNA binding protein that acts as a repressor of *BDNF* expression, and the level of acetylated histone H4 at the promoter, which contributes to the activation of *BDNF* promoter III, in response to forskolin treatment. We found that activation of the cAMP/PKA pathway leads to the dissociation of MeCP2 from *BDNF* promoter III and to an increase in acetylated H4 at the promoter. Importantly, these epigenetic modifications are inhibited when RACK1 levels are reduced by AdV-siRACK1. Taken together, these data suggest that RACK1 regulates *BDNF* promoter III activation via

interaction with the chromatin complex leading to MeCP2 dissociation (i.e., DNA demethylation) and histone H4 acetylation, thus resulting in increased *BDNF* expression. (1) Yaka et al (2003) J Biol Chem 278: 9630-8

2790/B504

Chromatin Control of CRE-Regulated Recombination and Gene Activation.

K. Ohta[1,2], K. Hirota[2,1]; [1]Dept of Life Sciences, Univ. of Tokyo, Tokyo, Japan, [2]RIKEN, Saitama, Japan

Objective: Chromatin regulates transcription, replication, repair, and recombination. The S. pombe meiotic recombination hotspot *ade6-M26* is activated by a CREB/ATF-type transcription factor on a cAMP responsive element (CRE)-like DNA element. We have been demonstrating that chromatin plays crucial roles in the activation of *M26* recombination hotspot. In this study, we investigate the molecular mechanisms of chromatin modification around CRE-related sequences and its roles in recombinational and transcriptional activation. Methods: Using various mutants and chromatin immunoprecipitation method, we have investigated status of local chromatin structure, histone modification, and binding of transacting factors. Results: We found that *M26* recombination hotspot is activated by the coordinate actions of various chromatin remodelers followed by the binding of the DSB recombinase Rec12/Spo11. Interestingly, the similar DNA sequences are found in stress-response genes and contribute to stress-dependent gene activation. We also demonstrate that a CRE-dependent stepwise chromatin remodeling occurs at *fbp1*$^{+}$ locus, as cells are faced to glucose starvation, in accordance with the appearance of a couple of long mRNA-type noncoding RNAs. Conclusions: CRE-related sequences play important roles in local chromatin dynamics to regulate both transcriptional and recombinational activity.

2791/B505

Characterization of DSB Repair in Heterochromatin.

I. Chiolo[2,1], H. Le[2,1], j. peng[1,2], G. Karpen[1,2]; [1]Molecular and Cell Biology, UC Berkeley, Berkeley, CA, [2]Genome Biology, LBNL, Berkeley, CA

Heterochromatin comprises 20-30% of many eukaryotic genomes, including flies and humans. Specific histone modifications are associated with heterochromatin, and its compact structure is stabilized by the binding of HP1 and the activity of Su(var)3-9 H3K9 methyltransferase. Given the chromatin compaction and abundance of repetitive sequences, DNA repair might be particularly challenging in heterochromatin. Heterochromatin has been characterized as recombinationally silent during meiosis, suggesting that at least some recombination pathways are prevented in this compartment. Previous studies in mammalian cells indicated that phosphorylation of the histone variant H2Ax, an early response to DSBs, is not observed in HP1-containing regions after irradiation (IR). Moreover, HP1 mobilization at the DSB sites is required for H2Ax phosphorylation, suggesting that DNA repair might require heterochromatin remodeling. We are characterizing how DSBs are processed in heterochromatin and if the recombination machinery is proficient for DNA repair therein. We use flies as model system, where heterochromatin has been very well studied. We observe that IR treatment induces the transient formation of pH2Av foci in heterochromatin. On the other hand, recombination foci are not formed within the heterochromatic compartment and they accumulate at the edge of the HP1 domain. The tagging of Rad51 and HP1 with fluorescent epitopes, combined with life imaging techniques, reveals that the HP1 domain is reorganized after IR and its mobility correlates with the dynamic reorganization of recombination foci. Finally, the depletion of Su(var)3-9 and other H3K9 methyltransferases by RNAi results in the formation of recombination foci in heterochromatin. Su(var)3-9null mutant flies are sensitive to IR, suggesting that recombination within the heterochromatic compartment might be source of genome instability. These results suggest that the heterochromatic structure prevents homologous recombination inside the heterochromatic compartment and that heterochromatic DSBs might be repaired by recombination only after removal from the original location and local displacement of HP1.

2792/B506

The JIL-1 Histone H3S10 Kinase Regulates Chromatin Structure by Multiple and Partially Redundant Mechanisms in *Drosophila.*

W. Cai, X. Bao, H. Deng, W. Zhang, J. Girton, J. Johansen, K. M. Johansen; Biochemistry, Biophysics & Molecular Biology, Iowa State University, Ames, IA

The JIL-1 tandem kinase in *Drosophila* localizes specifically to euchromatic interband regions of polytene chromosomes, is upregulated two-fold on the male X chromosome, and is the predominant kinase controlling the phosphorylation state of histone H3S10 at interphase. JIL-1 can be divided into four main domains including a NH2-terminal domain (NTD), the first kinase domain (KDI), the second kinase domain (KDII), and a COOH-terminal domain (CTD). Our results demonstrate that the CTD of JIL-1 is necessary and sufficient for correct chromosome targeting to autosomes, but that both COOH- and NH2-terminal sequences are necessary for upregulation on the male X chromosome. We furthermore provide evidence that a small 53 amino acid region within the CTD domain can interact with the tail-region of histone H3 suggesting that this interaction is necessary for the correct chromatin targeting of the JIL-1 kinase. Interestingly, our data indicate that histone H3S10 phosphorylation is not required for maintaining proper polytene chromosome morphology and band/interband organization and that the CTD alone is sufficient to rescue *JIL-1* null mutant polytene chromosome defects including those of the male X chromosome. Nonetheless, we also found that a construct (ΔCTD) which was without the CTD domain but retained histone H3S10 kinase activity and which could be localized to chromatin by sequences in the NTD was able to rescue autosome as well as partially rescue male X polytene chromosome morphology. Taken together these results indicate that JIL-1 may participate in regulating chromatin structure by multiple and partially redundant mechanisms. That JIL-1 kinase activity is one of these mechanisms is supported by the finding that histone H3S10 phosphorylation is necessary for preventing the spreading of the centromeric heterochromatin markers HP1 and H3K9me2 to the chromosome arms. (Supported by NIH grant GM62916).

2793/B507

DNA Repair Following Laser Micro-irradiation of Mitotic Chromosomes in PTK2 Cells.

V. Gomez-Godinez[1], L. Liaw[1], C. S. Lee[1], N. M. Wakida[1], L. Shi[2,1], V. Garces-Chavez[1], M. W. Berns[1,2]; [1]Beckman Laser Institute, University of California-Irvine, Irvine, CA, [2]Department of Bioengineering, University of California-San Diego, La Jolla, CA

Laser induced DNA damage is generally performed by exposing a linear sub-micron region within the nucleus. However, chromosomes in live mitotic cells can also be targeted with the laser. For example, in 1969 Berns et al. first demonstrated that a chromosome arm could be damaged during mitosis with a focused argon ion laser. Subsequent transmission electron microscopy (TEM) studies found that the irradiated region had changed in electron density. In 2002, Rieder et al. observed that the phosphorylation of histone H2AX on Ser 139, a phosphorylation specific to DNA double strand breaks, occurs when mitotic chromosomes are irradiated with a 532nm nanosecond Nd-YAG laser. Recently we observed that chromosomes irradiated with the 200 femtosecond near-infrared and the nanosecond green (532nm) laser immediately showed phase contrast lightening at the site of exposure followed by the formation of a phase dense spot by 30 seconds. The formation of this phase dark spot over time led us to believe that we might be observing the recruitment of DNA damage repair factors. To test this hypothesis we immuno-stained for initial DNA-damage responders (Rad50, Nbs1, and YH2AX). The immunostaining revealed that DNA damage repair factors Nbs1 and Rad50 co-localize to the phase dark spot. In addition, TEM was used in order to visualize the ultra-structure of the accumulating phase dark material. The TEM results demonstrate that the phase dark spot occurring at the irradiation site on the chromosomes corresponds to an accumulation of electron dense material at the damage site. Based on both the immunostaining and the TEM results, it appears likely that the phase dark material represents the accumulation of proteins involved in the repair and recognition of damaged DNA.

2794/B508

Role of Chromatin Protein Hmgb1 in UV-Light-Induced DNA Damage to the Retina.

G. Hoppe, J. E. Sears; Cole Eye Institute, Cleveland Clinic, Cleveland, OH

Hmgb1 is an abundant nuclear protein that regulates chromatin structure. In contrast to other tissues, expression of Hmgb1 in retinal photoreceptors undergoes substantial diurnal changes. We investigated whether protection of photoreceptor DNA from light-induced damage is one of the crucial functions of Hmgb1 in the retina. Immunohistochemistry was performed on the eyes of Long-Evans rats entrained to cyclic light or on cultured neuronal cell lines B35 and SK-N-SH using polyclonal antibody for Hmgb1 and monoclonal antibodies recognizing DNA lesions, e.g., cyclobutane pyrimidine dimers (CPD) and 8-oxo-7,8-dihydroguanine (8oxoG). Rhythmic expression of Hmb1 in rat retinal photoreceptors coincided with light phase, but could be also induced by light during dark phase after a 6-hr lag period suggesting that diurnal expression of Hmgb1 was entrained by light. Levels of CPD, characteristic for direct UV-induced DNA damage, were increased in light-exposed retina. Light-dependent elevation of CPD was found only in photoreceptor nuclei, but not in other retinal neurons. SK-N-SH cells exhibited variable levels of nuclear Hmgb1, presumably a cell-cycle-dependent phenomenon. When challenged with UV light, the rates of CPD formation were greater in the nuclei of cells, in which Hmgb1 was downregulated. Inversely, high levels of Hmgb1 appear to protect cultured neurons from direct DNA damage by UV. However, indirect DNA damage due to oxidative modification exemplified by 8oxoG was insensitive to the levels of Hmgb1. We conclude that the loss of Hmgb1 protein increases susceptibility of cultured neurons to direct DNA damage by UV light. Correlation between light-induced DNA lesions and levels of Hmgb1 in photoreceptor nuclei suggest a unique genoprotective and therefore neuroprotective role for Hmgb1 in retina.

2795/B509

A Positive Cooperation between Chromatin Remodeling and Histone Modifications in DNA Double Strand Break Repair.

H. Lee, S. Kim, J. Park, M. Kwon, J. Kwon; Ewha Womans University, Seoul, South Korea

Although mammalian SWI/SNF chromatin remodeling complexes have been well established to play important role in transcription, their role in DNA repair has remained unexplored. Recently, our laboratory has shown that mammalian SWI/SNF complexes are directly implicated in DNA double strand break (DSB) repair. However, the mechanism underlying these functions has remained unknown. Here, we found that BRG1, the catalytic core subunit of SWI/SNF complex, binds to the chromatin at DSBs in the manner that is dependent on the phosphorylation of H2AX. The phosphorylation of H2AX is required for acetylation of histone H3, and inversely, the acetylation of H3 is also required for the H2AX phosphorylation. Importantly, these interdependent histone modifications of H2AX and H3 occur specifically on the chromatin at DSBs in the fashion that requires the presence of SWI/SNF. We also found that the binding of BRG1 to the DSB-surrounding chromatin is mediated by the interaction of its bromodomain with acetylated H3, but not by the interaction with phosphorylated H2AX. These results suggest that there exists a novel intermolecular histone crosstalk for DSB repair, in which H2AX phosphorylation and H3 acetylation are stimulated by each other in a chromatin remodeling-mediated positive activation loop.

2796/B510

Identification of Chromatin-Associated Proteins of Numa, a Protein Involved in Chromatin Organization.

S. Jayaraman[1,2], P. C. Abad[1], J. Galan[3], A. W. Tao[3], C. V. Stauffacher[2], S. A. Lelievre[1]; [1]Basic Medical Sciences, Purdue University, West Lafayette, IN, [2]Biological Sciences, Purdue University, West Lafayette, IN, [3]Biochemistry, Purdue University, West Lafayette, IN

Several publications have linked the large coiled-coil nuclear protein NuMA with cell differentiation and cancer development. However, the mechanisms by which NuMA controls cell phenotype are not known. We have previously shown that NuMA is abundantly present in the chromatin compartment and that the distal portion of NuMA-C terminus (CT) shares sequence similarity with chromatin-associated proteins. Both antibody-induced alteration of NuMA distribution and siRNA-mediated silencing of NuMA expression modified higher order chromatin organization in non-neoplastic breast epithelial cells, which in turn, inhibited proper differentiation of these cells into glandular tissue structures. To further understand how NuMA controls chromatin structure, we sought to identify interacting partners of NuMA. NuMA cofractionated, co-immunoprecipitated and colocalized with the ATPase of the ATP-dependent chromatin remodeling ISWI complex family in non-neoplastic breast epithelial cells, suggesting that NuMA may influence chromatin remodeling. To unravel other potential chromatin-associated partners of NuMA, immunopecipiations (IPs) were performed from HeLa cell extracts using three different antibodies targeting either the globular N-terminus (NT), globular CT, or central coiled-coil region (CC). IPs with nonimmune buffers or nonspecific immunoglobulins were used as controls. All samples were trypsin-digested and subjected to electron spray ionization mass spectrometry. Proteins found only in the immune sample and not in any of the eight controls were retained including 1, 50, 102 proteins specific for IPs performed with antibodies targeting the NT, CT and CC domains, respectively. Notably, out of the five proteins commonly found in IPs

performed by targeting the CC and CT regions was a protein from a chromatin remodeling complex that contains the ATPase previously identified to interact with NuMA. Studies are ongoing to investigate the potential interaction between NuMA and such chromatin remodeling complex during breast epithelial differentiation. (*) Jayaraman and Abad contributed equally to this work.

2797/B511

Morphological and Immunocytological Characterization of Snake-like Chromatin Epithelial Cells from the Conjunctiva of Patients Suffering from Dry Eye.

K. Jirsova[1,2], K. Juklova[1]; [1]Laboratory of the Biology and Pathology of the Eye, Institute of Inherited Metabolic Disorders, First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic, [2]Ocular Tissue Bank, General Teaching Hospital and First Faculty of Medicine, Charles University in Prague, Prague, Czech Republic

Objectives: The aim of our study was a detailed morphological and immunocytological description of snake-like chromatin (SLC) structures to elucidate the mechanisms by which SLC arises in epithelial cells of the human conjunctiva. Methods: Impression cytology specimens from the bulbar conjunctiva of 15 patients with dry eye syndrome and 10 control specimens were used. The imprints were harvested bilaterally on nitro acetate cellulose filter papers and on Millicell membranes. The cell morphology and the number of micronuclei (MN) were assessed. Proliferation markers (Ki-67 and PCNA), nucleolar proteins (fibrillarin and B23) and components of the nuclear lamina (lamin A/C and B) were detected using indirect immunofluorescence. Apoptotic cells were determined morphologically, using the TUNEL technique and by active caspase-3 detection. Results: Different stages of SLC cells (membrane notching, cuff constricted SLC structures and binucleate forms) were found in pathological specimens. No SLC structures were present in healthy subjects. The number of MN in control epithelial cells reached 1.3 /1000 cells, 22.1 in SLC-positive cells and 3.5 in SLC-negative cells in the pathological specimens. Changes in the location of the signal (nonrandom redistribution into spots closely adjectant to SLC structures) revealed the nucleolar protein fibrillarin. B23, Ki-67 and PCNA positivity was noted in SLC-negative cells, but never in SLC-positive cells. The absence of lamin A/C in SLC-positive cells compared to SLC-negative epithelial cells was observed. The attenuation of signal staining in SLC-positive cells compared to SLC-negative cells revealed lamin B. No more than 6% of all SLC-positive cells, as assessed by different methods, revealed apoptotic changes. Conclusions: The high level of MN structures in SLC cells, without the presence of characteristic mitotic markers but with an abnormal nucleus with membrane notching and binucleated SLC forms, leads to the conclusion that the phenomenon of SLC is a special type of nuclear/cell division similar to amitosis. Our results indicate that SLC is not a form of apoptosis, but that cells with snake-like chromatin may be affected by apoptosis.

2798/B512

***Brambleberry* Functions during Cleavage Stage to Maintain Nuclear Integrity.**

E. W. Abrams, F. L. Marlow, L. Kapp, T. Gupta, M. C. Mullins; Cell and Developmental Biology, University of Pennsylvania, Philadelphia, PA

Embryonic development in most animals initiates with a series of rapid and synchronous cell divisions. These rapid cell divisions, referred to as the cleavage cycles, lack cell cycle checkpoints. An understanding of how chromosome integrity is maintained during this very critical period of metazoan development is currently lacking. In a forward genetic screen in zebrafish, we recently identified a maternal-effect mutant, *brambleberry* (*bmb*), which exhibits profound chromatin defects during the cleavage stage of development. In *bmb* mutants, a typical interphase nucleus appears as a cluster of chromatin bodies that is sometimes peripherally associated with a few DAPI-positive micro-fragments. This is in contrast with wild-type interphase nuclei, which display a single, large chromatin body. Examination of the mitotic spindle indicates that *bmb* chromosomes are not properly aligned on the metaphase plate and are more dispersed compared to wild-type controls. Interestingly, the microtubule spindle appears to function normally during mitosis and, in most cases, is able to attach to wayward chromosomes. Inspection of the nuclear envelope, by staining for members of the nuclear pore complex, reveals that during interphase *bmb* chromatin bodies appear individually encased in nuclear envelopes, whereas in wild-type controls interphase nuclei are contained within a single nuclear envelope. To our knowledge this constitutes a novel nuclear envelope phenotype. Surprisingly, *bmb* nuclear morphology becomes progressively improved just after the mid-blastula transition, when zygotic transcription initiates, despite mutants failing to undergo cell movements associated with normal development at this point. This effect is not diminished when both maternal and paternal functions of *bmb* are eliminated, suggesting that *bmb* functions specifically during the cleavage stage of embryonic development to maintain nuclear integrity. Further characterization of *bmb* mutants involves examining other mitotic components and aspects of nuclear/chromatin structure. Finally, significant effort is being put forth into the positional cloning of the *bmb* gene to elucidate its molecular function.

2799/B513

SIRT1 Inhibitor Alleviates FMR1 Gene Silencing in Fragile X Cell Lines.

R. Biacsi, D. Kumari, K. Usdin; LMCB, NIH/NIDDK, Bethesda, MD

Fragile X syndrome, the most common heritable form of intellectual disability, is caused by expansion of a CGG*CCG-repeat tract in the 5' untranslated region of the FMR1 gene to >200 repeats, and subsequent gene silencing. The silenced allele can be reactivated with 5-aza-2-deoxycytidine. However, this compound is toxic and requires DNA replication for its efficacy. Thus, it may not be useful in post-mitotic cells like neurons where the consequences of gene silencing is most apparent. Histone deacetylase (HDAC) inhibitors, like Trichostatin A and Phenylbutyrate which target the Class I, II and IV HDACs have little or no effect on FMR1 expression. However, we found that inhibitors of the Class III HDACs, like splitomicin, can cause significant gene reactivation. We showed that splitomicin acts by inhibiting deacetylation of lysine 16 of histone H4 by the SIRT1 protein deacetylase. This favors the activity of hMOF, the human H4K16 acetylase. Gene reactivation occurs without DNA demethylation. Thus H4K16 deacetylation is a downstream consequence of DNA methylation and is a late step in the silencing process. Since DNA replication is not required for splitomicin-mediated gene reactivation, this class of drugs may be more useful than DNA methylation inhibitors for the reactivation of FX alleles in neurons.

Tissue Specific Gene Expression (2800 – 2824)

2800/B514

The Drosophila LEM Domain Proteins, Bocksbeutel and Otefin, Make Over-lapping Contributions to Nuclear Lamina Function.

S. R. Wilmington[1], E. E. Hornick[1], B. S. Pinto[2], L. L. Wallrath[1], P. K. Geyer[1]; [1]Department of Biochemistry, University of Iowa, Iowa City, IA, [2]MCB Program, University of Iowa, Iowa City, IA

Proteins in the nuclear envelope and underlying lamina establish a network of interactions that provide mechanical stability to the nucleus and contribute to the regulation of nuclear trafficking, signal transduction and gene expression. One family of nuclear lamina proteins possesses an ~ 40 amino acid motif, known as the LAP2 emerin MAN1 domain (LEM-D), that associates with the chromatin binding protein Barrier-to-Autointegration Factor (BAF). LEM domain proteins have redundant lamina functions that may involve anchoring chromatin to the nuclear periphery. Loss of LEM-D proteins causes several tissue-specific human diseases, including muscular dystrophy, cardiac conductance diseases and a bone density disorder, even though these proteins are globally expressed. The Drosophila genome encodes two LEM-D proteins that share sequence conservation with emerin, named Bocksbeutel (Bocks) and Otefin (Ote). Similar to emerin, these proteins are serine/threonine rich, accumulate in all tissues throughout development and are enriched at the nuclear periphery. To define the roles of the Drosophila emerin homologues, genetic analyses were undertaken. Null alleles of *bocks* were generated by imprecise *P* excision. Flies lacking Bocks are viable and fertile, with no visible phenotypes. Several null alleles of *ote* were identified among existing Drosophila mutant collections. Flies lacking Otefin are viable, with females having rudimentary ovaries that display defects in differentiation of germline stem cells. The female sterility associated with *ote* mutants is partially rescued by over-expression of Bocks, suggesting that the Drosophila emerin homologues share functions. This postulate is further supported by observations that *bocks ote* double mutants do not survive to adulthood. Taken together, our data indicate that Drosophila LEM domain proteins make distinct and over-lapping tissue-specific contributions to lamina function.

2801/B515

DDC Deregulates the Expression of Non-Coding RNA Which Is Prevented by Feeding Same.

J. Oliva; LA Biomed, Torrance, CA

Background: Mice fed DDC (0.1%) for 10 weeks, and then withdrawn from the drug for 1 to 4 months, reform Mallory-Denk Bodies (MDBs) when the drug is refed for 7 days. The liver cells that form MDBs increase in number and partially replace normal liver cells when the MDBs form. This is prevented by S-Adenosylmethionine (SAMe) when fed with the DDC. Method: Microarray analyses were done on RNA from livers of the mice refed DDC and DDC+SAMe. Real-time PCR was used to study the expression of non-coding RNA (ncRNA). Results: Microarray analysis of mice livers showed that MDB formation was associated with changes in the expression of a large number of genes. Microarray analysis showed that DDC induced the expression of H19 and antisense transcript (AIR) by 2 and 9 fold respectvely, and down regulated the expression of GTL2 by 8 fold. SAMe prevented this deregulation of the expression produced by DDC. The results were also measured using Quantitative RT-PCR for the drug fed and drug primed mice, which were refed the drug for 7 days. H19 and AIR were induced by 60 and 30 fold respectively, and GTL2 was reduced by 4 fold. SAMe prevented the deregulation induced by DDC. At this time MDB formation was observed. Conclusion: DDC is able to regulate the ncRNA expression of H19, Air and GTL2. When drug primed mice were refed DDC with S-adenosylmethionine (SAMe) (4g/kg body weight), the deregulation of these ncRNAs are prevented. The three ncRNAs found are imprinted genes, involved in many diseases such as hepatocarcinoma, renal carcinoma and neuroblastoma. The question remaining is: are the changes in the expression of ncRNAs involved in the mechanism of MDB formation? Supported by a grant NIH/NIAAA 8116 and Alcohol Center Grant on liver and pancreas P50-011999, morphology core.

2802/B516

Sperm and Tissue-Dependent Differential Gene Expression in the Reproductive Tract of the Turkey (Meleagris Gallapovo) Hen.

O. T. Foye-Jackson[1], J. L. Long[1], M. R. Bakst[1], L. A. Blomberg[1], K. G. Becker[2], W. H. Wood[2], J. P. McMurtry1[1]; [1]Animal Biosciences and Biotechnology Lab, United States Dept of Agriculture, ARS, Beltsville, MD, [2]Gene Expression and Genomics Unit, Resources Branch, National Institute of Health-National Institute on Aging, Baltimore, MD

Similar to all birds, the reproductive tract of the turkey hen contains specialized epithelial invaginations within the uterovaginal (UVJ) junction known as sperm storage tubules (SST). The SST microenvironment maintains viable turkey sperm that can fertilize successively ovulated ova for up to 10 weeks after a single insemination; however, current in vitro methodologies can't sustain the fertilizing capability of turkey sperm beyond 6-8 h. This project aims to elucidate the molecular and cellular mechanisms involved in regulating long-term sperm storage in the reproductive tract of turkey hens. Suppression Subtractive Hybridization was utilized to determine differential gene expression between the SST and vaginal epithelium (VE). Equimolar amounts of total RNA were pooled from the SST and VE tissues of ten 38-week old turkey hens recovered 48h post-insemination and used to construct cDNA libraries. Gene annotations were determined utilizing the Chicken Genome database NCBI. Validation was determined by quantitative real-time PCR (qrPCR) analysis. Approximately 9% of the total transcripts (480) were identified as differentially expressed to the SST of AI hens. Expression levels of 17 genes were determined by qrPCR analysis of the original 10 VE and SST samples. Avidin was the only gene examined that demonstrated differential expression in SST tissue, with a 40-fold ($p<0.05$) increase compared to VE. Also, there was 9-fold increase in mRNA expression of avidin-related protein 2 in the SST versus the VE ($p<0.05$). In a separate set of experiments the SST of sham and artificially inseminated (AI) hens were examined for avidin expression. Significant increases ($p<0.05$) in mRNA expression of avidin (9-fold) and avidin-related protein 2 (4-fold) occurred in the SST of AI compared to sham hens. When comparing non-inseminated (NI) and AI hens, avidin protein was found to be expressed in the UVJ surface epithelium in both groups; however protein expression was higher in NI hens. Interestingly, the SST of control hens had greater avidin protein expression than SST of AI hens by immunohistochemical analysis. These results suggest that avidin is differentially regulated in the reproductive tract of turkeys.

2803/B517

The Transcriptional Regulation of Aurora-C in Spermatogenesis and Tumorigenesis.

L. Hung[2,1], J. Tsou[2,1]; [1]Department of Pharmacology, National Cheng Kung University, Tainan, Taiwan, [2]Institute of Biosignal Transduction, Tainan, Taiwan

Aurora is a family of highly conserved serine/threonine protein kinases. There are three aurora members in the mammals: Aurora-A, -B and -C. Aurora-A and -B are expressed in many cell lines and tissues, and played essential roles in regulating many processes during cell cycle. Less is known about Aurora-C kinase, which is specifically and highly expressed in the testis. During meiosis, Aurora-C kinase shows a dramatic movement and behaves as a chromosomal passenger protein and interacts with INCENP and survivin directly, suggesting that Aurora-C kinase may play a role similar to Aurora-B kinase in the cell. Interestingly, Aurora-C has been found overexpressed in primary colorectal cancers and thyroid carcinoma and in several cancer cell lines. However, the reason and effect of overexpressed Aurora-C in cancer cells are not characterized. In the present study, we demonstrated that the mouse Aurora-C promoter activity in germ-line cell line - GC-1 is higher than the somatic cell line - NIH3T3 cells. By 5'-serial deletion assay, two negative regulatory cis-elements of the mouse Aurora-C promoter were identified. The detailed molecular mechanism of overexpressed Aurora-C in tumors is currently investigation.

2804/B518

Regulation of Chondrocyte Hypertrophy by SOX9 in a Novel Transgenic Mouse Model.

B. Liang[1], D. Chen[1], T. Pizzuto[1], B. Lee[2], G. Zhou[1]; [1]Orthopaedics, Case Western Reserve University, Cleveland, OH, [2]Molecular and Human Genetics, Baylor College of Medicine, Houston, TX

The disturbed balance in cartilage metabolism plays an important role in the pathogenesis and progression of osteoarthritis (OA). Transcription factors are key regulators of cartilage metabolism by stimulating chondrogenesis under both physiologic and pathologic conditions. Among them, SOX9 is essential for successive steps of chondrogenesis while RUNX2 is a positive regulator of chondrocytes maturation. RUNX2 also represses adipocytes differentiation and its deficiency in chondrocytes leads to increased adipogenesis. We have previously shown that SOX9 can directly interact with RUNX2 and inhibit its transcriptional activity in vitro. To directly investigate SOX9 effects during chondrocytes hypertrophy, a novel Col10a1-SOX9 transgenic mouse model was generated in which SOX9 is specifically expressed in hypertrophic chondrocytes driven by a 10kb Col10a1 promoter. In monolayer culture of rib hypertrophic chondrocytes isolated from 6-day-old mice, expression of Col10a1, a downstream target of Runx2, was decreased by 55% in transgenic mice by quantitative RT-PCR. In comparison with wild-type mice, Col10a1-SOX9 hypertrophic chondrocytes culture also displayed increased adipogenesis with more lipid droplets accumulation by Oil Red-O staining and higher expression levels of adipogenic genes. Additionally, mineralized areas of hypertrophic cartilage were significantly reduced by 10% ($p<0.05$) in proximal tibia at 10-week-old Col10a1-SOX9 transgenic mice. Interestingly, 11-month-old knee joints of Col10a1-SOX9 mice but not wild-type littermates developed spontaneous osteoarthritis chararistic features, including joint space narrowing, development of osteophytes, and subchondral sclerosis as assayed by X-ray radiography. Moreover, Safranin-O histology revealed severe degradation of articular cartilage and bony outgrowth in 6-month- and 11-month-old transgenic mice but not in wild-type controls. In transgenic mice joints, the surfaces of the articular cartilage become rough and disorganized. Therefore, our study not only confirms the direct inhibitory effect of SOX9 on chondrocyte hypertrophy during cartilage formation, it also reveals a potential novel role of SOX9 in osteoarthritis pathogenesis.

2805/B519

C/EBPβ (LAP*) Down-regulates the Ric-8b Gene Expression in Bone Cells.

R. Grandy, J. Olate, M. Montecino; Department of Biochemistry and Molecular Biology, Faculty of Biological Sciences, University of Concepcion, Concepcion, Chile

The Ric-8 gene encodes a guanine-exchange-factor (GEF) protein that regulates G protein- mediated cellular signaling processes. Ric-8 was first described in the nematode C.elegans, regulating the liberation of synaptic vesicles as well as the precise positioning of the mitotic spindle during asymmetric cell division. In mammals, two Ric-8 homologous genes have been found (Ric-8a and Ric-8b) and recent studies suggest complementary roles for each encoded protein. The expression of both genes has been reported in various tissues although are predominantly expressed in the nervous system. We found that Ric-8b is expressed also in osteoblastic cells and that this expression varies during osteoblast differentiation. We performed an in silico analysis of the Ric-8b gene promoter and detected putative binding sites for the transcription factor C/EBPβ, whose expression and activity have been also reported in osteoblastic cells. Because this transcription factor exhibits three isoforms with different transcriptional activities we assessed their contribution to the Ric-8b promoter activity in osteoblastic cells. 2 Kb of the Ric-8b gene promoter were first cloned in front of a luciferase reporter gene and then its activity evaluated in transient transfection studies. The response of the Ric-8b promoter to the different isoforms of C/EBPβ (LAP*, LAP and LIP) was also determined. Using different promoter deletions in transient expression studies and chromatin immunoprecipitation assays we determined the Ric-8b gene promoter region that is recognized by C/EBPβ in osteoblastic cells. Our results demonstrate that Ric-8b is expressed in pre-osteoblastic cells and that this expression is repressed as the differentiation process progresses. In addition, we show that the minimal Ric-8b promoter activity resides within 500 bp upstream the transcriptional start site. Finally, we demonstrate that the long C/EBPβ isoform (LAP*) represses the Ric-8b promoter activity in osteoblastic cells. These results support a new regulatory role of C/EBPβ (LAP*) as a repressor during osteoblast differentiation, in contrast with its well-established function as a transcriptional activator.

2806/B520

Regulation of Differentiation and Migration of Parathyroid during Development by Transcription Factor MafB.

A. Kamitani[1], M. Hamada[2], M. Miyai[1], T. Moriguchi[3], S. Takahashi[2], K. Kataoka[1]; [1]Nara Institute of Science And Technology, Ikoma, Nara, Japan, [2]Univ. Tsukuba, Tsukuba, Japan, [3]Tohoku Univ., Sendai, Japan

Parathyroid glands play a central role in the regulation of the extracellular calcium homeostasis. These glands detect decrease of the blood calcium levels by cell surface calcium-sensing receptor (CaSR) and secrete parathyroid hormone (PTH. In mice, development of the parathyroid glands begins at embryonic day 10.5 (E10.5) as bilateral primordium common to the thymus in the third pharyngeal pouch

endoderm, and then the parathyroid primordium begins to express the PTH at E11. By E13.5, the parathyroid and thymus primordia migrate together toward the heart, and they separate into a parathyroid gland and a thymic lobe, respectively. Subsequently, the parathyroid migrates to the thyroid gland that is the eventual adult locations. However, the molecular mechanisms that control the parathyroid development and PTH expression are not understood. We previously found that the transcription factor MafB was expressed in the parathyroid cells. In this study, we investigate the roles of transcription factor MafB in parathyroid glands by using MafB-knockout (KO) mice. As the MafB homozygous embryos die shortly after birth, we investigated the parathyroid glands at E18.5. The normal parathyroid glands are located near the thyroid glands. In contrast, MafB-KO embryos lacked the parathyroid glands in thyroid, but they had ectopic parathyroid-like cells in the thymus. In MafB heterozygous embryos, such parathyroid-like cells were located between the thyroid glands and the thymus. PTH expression was significantly decreased in these parathyroid-like cells in MafB-KO embryos. As far as we have examined, expression of P-cadherin and EphA1 receptor, which are involved in cell adhesion and cell-cell repulsion, was significantly reduced in MafB homozygous mice. Taken together, these observations suggest that MafB regulates the PTH expression, as well as migration of the parathyroid glands during their developmental processes through in inducing P-cadherin and EphA1 expression.

2807/B521

Krüppel-like Factor 4 (KLF4) Positively Regulates Human Ghrelin Expression.

M. H. Jung; Pusan National University, Busan, South Korea

Ghrelin, an endogeneous ligand for the GH secretagogue receptor, exerts a variety of metabolic effects including GH secretion, food intake, energy balance, insulin secretion and adipogenesis. Although ghrelin exhibits a variety of biological function, the mechanism regulating ghrelin expression is nearly unknown yet. Ghrelin is expressed in gastrointestinal tissues, predominantly stomach. Krüppel-like factor 4 (KLF4) is a zinc finger transcription factor that is also highly expressed in gastrointestinal tract. Therefore, we hypothesized that ghrelin expression may be associated with KLF4. In this study, we showed that tissue distribution of ghrelin expression was in accordance with that of KLF4 expression. Furthermore, treatment of butyrate, inducer of KLF4 expression, stimulated ghrelin expression, and fasting which induces ghrelin expression also increased KLF4 expression, suggesting ghrelin expression have a potential relationship with KLF4. Then, we investigated the effects of KLF4 on promoter activity of human ghrelin gene, and determined KLF4 responsive region in the promoter. Expression of KLF4 specifically stimulated activities of two human ghrelin promoters for short or long transcript in dose dependence in AGS stomach cells. However, this stimulation was not seen with a mutant KLF4 construct. Transfection studies using 5' deletion mutant constructs of human ghrelin promoter revealed that KLF4 responsive element locates between -1228 and -1105 region. Electrophoretic mobility shift assay using oligonucleotide containing -1165/-1146 demonstrated KLF4 binding to human ghrelin promoter. Deletion of the -1165/-1146 region abrogated KLF4-induced transactivation of ghrelin promoter. Collectively, these results indicated that KLF4 can act to regulate human ghrelin gene expression positively through binding to KLF responsive region in the promoter

2808/B522

Expression of PPARγ Gene Is Repressed by DNA Methylation in Adipose Tissue of Diabetes Model Mice.

K. Fujiki[1], F. Kano[1], K. Shiota[2], M. Murata[1]; [1]Department of Life Sciences, Graduate School of Arts and Sciences, the University of Tokyo, Tokyo, Japan, [2]Department of Animal Resource Sciences, Graduate School of Agricultural and Life Sciences, the University of Tokyo, Tokyo, Japan

Adipose tissue works not only as the storage of energy as lipid, but also as the endocrine tissue that regulates metabolic activities of the organism by secreting various kinds of hormones. Peroxisome Proliferator Activated Receptor γ (PPARγ) is a key regulator of the adipocyte differentiation, which induces the expression of the adipocyte specific genes in pre-adipocytes and differentiates them into adipocytes. Furthermore, PPARγ has an important role to maintain the physiological function of the mature adipocyte properly. In this study, we investigated the epigenetic transcriptional regulation system in the expression of PPARγ gene, especially by DNA methylation, and the relevance of this system to the pathogenesis of metabolic syndrome. In 3T3-L1 pre-adipocytes, the promoter of PPARγ gene was hypermethylated and, when induced to differentiate, a progression in demethylation of the promoter was observed, followed by an increase of mRNA expression. Moreover, 5'-aza-cytideine treatments broke down the silencing of the gene dose-dependently and in vitro methylated PPARγ promoter repressed the transcription of the downstream reporter gene, which further reinforced that the expression of PPARγ is under the control of its promoter methylation. One of the pathology of metabolic syndrome includes the decline in the activity of PPARγ. We next compared the methylation status of PPARγ promoter of WT mice to diabetes model mice, such as +Lepr db/+Lepr db mice and diet induced obesity mice, found a methylation excess in epididymal adipose tissue of the diabetes model mice. Consistent with this, a decrease in the level of PPARγ mRNA was observed in the diabetes model mice. These results suggested the possibility that the decline in the expression of PPARγ caused by the disturbance in the epigenetic system of the gene contributes the pathogenesis of metabolic syndrome.

2809/B523

Anti-Oxidative Effects of Korean Red Ginseng on Immobilization Stressed Mice and Oxidative Stressed SK-N-SH Cells.

M. Lee[1], E. Kim[1], S. Choi[2], D. Rhee[1]; [1]Pharmacy, Sungkyunkwan University, Suwon, South Korea, [2]Department of Molecular Science and Technology, Ajou University, Suwon, South Korea

Immobilization (IMO) stress has been implicated in motor activity, memory dysfunction, anxiety, pain perception and depression-like behaviors in animals. These adverse effects are mediated by stress-induced neurochemicals and hormonal abnormalities that are often associated with oxidative damage. Reactive oxygen species (ROS), mainly superoxide anion (O2-) and hydrogen peroxide (H2O2), may mediate oxidative stress. Oxidative stress is a characteristic feature in a number of neurodegenerative disorders. Overexpression of peptidyl arginine deiminase type IV, PADI4, induces apoptosis mainly through cell cycle arrest in G1 phase and a mitochondria-mediated pathway, suggesting an important role for PADI4 in immune cell differentiation and cell death. Ginseng, the root of *Panax ginseng* C.A. Meyer (Araliaceae), or Korean red ginseng (KRG) widely has been used as an anti-stress agent. However, effect of ginseng on gene expression, especially PADI4, in brain has not been reported. Here we elucidated molecular mechanisms of KRG on IMO stresses using the microarray and real-time PCR. Microarray analysis revealed that IMO stress induced PADI4 and SLC4A1 genes whereas they were repressed by KRG

pre treatment. Moreover, myeloperoxidase (MPO) activity and malondialdehyde (MDA) levels were increased on IMO stress condition but reduced in KRG pre treated mice brain. In addition, KRG pre-treatment was increased cell viability of SK-N-SH cells exposed to H2O2 than the non-pre treated control. Thus, our data suggest that KRG protects IMO stressed mice brain and oxidative stressed SK-N-SH cells. And PADI4 and SLC4A1 genes can be used as stress markers.

2810/B524

Gene Expression Profile in Placenta of Fetus with Intrauterine Growth Restriction.

M. Lee[1], Y. Jeon[1], Y. Kim[2], S. Jung[1]; [1]Biochemistry, Ewah Womans University, Seoul, South Korea, [2]Obstetrics and Gynecology, Ewah Womans University, Seoul, South Korea

The aim of this study was to investigate whether alteration of gene expression related with glucose metabolism is associated with intrauterine growth retardation (IUGR) and to evaluate the differences of glucose metabolism related enzyme level in the blood of pregnant women and cord blood between normal pregnancy and IUGR pregnancy. We used oligonucleotide microarray analysis to identify differentially expressed metabolic genes in placentas of fetus with IUGR compared with placentas of the control group. The expression of genes related to glucose metabolism was up-regulated in IUGR placentas including dihydrolipoamide S-acetyltransferase (E2 component of pyruvate dehydrogenase complex, DLAT), 6-phosphofructo-2-kinase/fructose-2, 6-biphosphatase 2 (PFKFB2), lactate dehydrogenase C (LDHC), oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide) (OGDH), insulin-like growth factor (IGF) II, and insulin (INS). In contrast, IGFI was down-regulated in IUGR placentas. In the IUGR group, the glucose concentrations were significantly decreased in maternal serum and in cord blood. However, insulin concentrations were increased in maternal serum and in cord blood. Moreover, IGF-II concentrations were higher in maternal serum and lower in cord blood. These results can provide an insight to a cross-talk between IUGR and metabolic disorders after birth.

2811/B525

The Effects of Proteosome Inhibition on the Levels of α-tubulin and S-crystallin in Light-adapted Octopus Retinas.

J. Castaneda, S. Kelly, L. Robles; Biology, CSU Dominguez Hills, Carson, CA

Vision impairment and blindness affect millions of people across the US. Many of these vision impairments are derived from inherited retinal diseases affecting the photoreceptors or other retinal cells. In photoreceptors and retinal pigment epithelial cells, mutations in cytoskeletal proteins lead to retinal degeneration. We have previously reported the differential expression of α-tubulin and S-crystallin in dark- and light-adapted octopus retinas (Zuniga et. al, 2003). Our current aim is to determine if decreased levels of α-tubulin and S-crystallin protein in the eyes of light-adapted octopus retinas are caused by ubiquitination and subsequent degradation by the proteosome. To determine if α-tubulin and S-crystallin are degraded in the light by the proteasome, octopuses extracts with the proteasome inhibitors received ocular injections of proteasome inhibitors after 3-4 hours of dark adaptation, and their eyes were subjected to dissection at 5-, 15-, 30-, 45-, 60-, 120-, and 180-minute intervals post light adaptation. Retinal extracts from a second set of octopuses sampled as previously described were treated by incubation of extracts with the proteasome inhibitors. Total retinal protein was isolated and equal concentrations were subjected to SDS-PAGE and Western blot analysis. Volumetric analysis of the extracts treated with proteasome inhibitors revealed significant increases in the levels of α-tubulin and S-crystallin protein in light-adapted retinas. Increases in the levels of α-tubulin and S-crystallin protein in light-adapted retinas after proteasome inhibition treatment suggest that decreased levels of these proteins in light-adapted retinas are a result of degradation by the proteasome.

2812/B526

SUZ12: Keeping in Check Non-Specific Differentiation Pathways in Intestinal Epithelial Cells.

C. Asselin, N. Turgeon, M. Blais, J. Delabre; Anatomie et Biologie Cellulaire, Université de Sherbrooke, Sherbrooke, QC, Canada

Polycomb-group (PcG) proteins form multimeric protein complexes involved in transcriptional silencing. The Polycomb Repressive complex 2 (PRC2) contains among others, the Suppressor of Zeste-12 protein (SUZ12) and the histone methyltransferase Enhancer of Zeste protein-2 (EZH2). This complex catalyzes the di- and tri-methylation of histone H3 lysine 27 (H3K27me2 and H3K27me3), and is essential for embryonic development and stem cell renewal. However, the role of Polycomb-group (PcG) proteins, including SUZ12, in intestinal epithelial cell (IEC) differentiation is not known. Thus, we verified the role of SUZ12 in intestinal epithelial cells. METHODS: We determined SUZ12 and EZH2 expression in intestinal protein fractions by Western blot. We depleted SUZ12 expression in the IEC-6 rat crypt-derived cell line with a shRNAi against SUZ12. We measured cell proliferation by MTT assays and analysed the expression of cell cycle regulators by Western blot. Microarray and semi-quantitative RT-PCR analysis were performed to determine the global role of SUZ12 in IEC-dependent gene expression. RESULTS: 1) SUZ12 and EZH2 were differentially expressed along the intestinal crypt-villus axis. 2) SUZ12 depletion led to a decrease in EZH2 expression and H3K27 tri-methylation in IEC-6 cells. 3) While cell proliferation decreased in the exponential phase of growth, SUZ12-depleted cells achieved higher cell densities. This correlated with increased cyclin D2 protein levels. 4) Microarray and RT-PCR analysis showed that SUZ12 depletion specifically increased neuronal and inflammatory gene expression, transcriptionnally and post-transcriptionnally. CONCLUSION: These results demonstrate that SUZ12, by keeping in check other differentiation pathways, allows the expression of the specific intestinal epithelial cell phenotype. Supported by CCFC.

2813/B527

Synergistic Activation of Atrial Natriuretic Peptide (ANP) Promoter by GATA4 and Myogenin.

M. Hasan[1], S. Miyamoto[1,2], R. Matsuoka[1], N. Kawaguchi[1]; [1]IREIIMS, Tokyo Women's Medical University, Tokyo, Japan, [2]Department of Cardiovascular Surgery, Tokyo Women's Medical University, Tokyo, Japan

The Zn finger containing GATA4, a tissue restricted transcription factor, regulates the cardiac specific gene expression through combinatorial interaction with semi restricted transcription factors such as MEF2c, NKx2.5 etc. or basic helix loop helix (bHLH) transcription factor such as dHAND. Though, Myogenin or MyoD has been recognized as skeletal muscle specific bHLH transcription factors, co-expression of

Myogenin or MyoD and GATA4 was observed in chick cardiac Purkinje fiber cells (Takebayashi-Suzuki K et al., Dev. Biol. 2001). However, importance of the co-expression of these two tissue restricted transcription factors is still unknown. Here we report for the first time the co-expression of GATA4 and Myogenin in cardiac stem cells (CSCs) isolated from adult rat hearts. We isolated c-kit positive CSCs from adult rat hearts using AUTOMACS and characterized their multi potency by checking the expression of various marker genes using RT-PCR and immunocytochemistry. Different biochemical approaches including, transfection, immunoprecipitation, EMSA, RNAi, reporter assay etc. were taken to carry out this study. We show that these two transcription factors bind each other and synergistically trans-activate the expression of GATA4 specific promoter of atrial natriuretic peptide (ANP) gene. This combinatorial function is independent of E-box but depends on the GATA sites present on the ANP promoter. Immunoprecipitation and EMSA suggest that these two proteins bind with each other and binding of Myogenin with GATA4 enhances the GATA4 promoter binding activity. Furthermore, C terminal Zn finger domain and bHLH region of GATA 4 and Myogenin, respectively, are required for this synergistic function of transcriptional activation. Interestingly, over expression of GATA4 causes the down regulation of Myogenin but GATA4 remains unaffected when Myogenin is over expressed suggesting GATA4 could act upstream of Myogenin and might regulate its expression. Taken together, we can conclude that these two lineage specific transcription factors GATA4 and Myogenin, as observed in cardiac stem cells, might play important role in regulating cardiac gene expression and heart development in vivo.

2814/B528

Regulation of Tissue Plasminogen Activator and Plasminogen Activator Inhibitor-1 in Lipopolysaccharide-Stimulated Rat Primary Neuron, Astrocyte and Microglia.

J. Kim[1], K. Kwon[1], S. Han[1], S. Yang[1], J. Lee[1], K. Ko[2], C. Shin[1]; [1]School of Medicine, Konkuk University, Seoul, South Korea, [2]Pharmacology, Seoul National University, Seoul, South Korea

Originally studied in blood clot dissolution process, a serine protease tissue plasminogen activator (tPA) has also been found in brain and implicated in the regulation of neural plasticity, neural development, neurotoxicity and glial activation. The enzymatic activity of tPA is regulated by an endogenous inhibitor plasminogen activator inhibitor-1 (PAI-1), which is a member of serpin (serine protease inhibitor) family. Because the function of tPA is mediated by both the enzymatic and cytokine-like effect of tPA, understanding the regulatory mechanism of both tPA and PAI-1 may provide more delicate way to modulate these essential regulators of brain fuinction. In this study, we investigated the synthesis and release of tPA and PAI-1 in lipopolysaccharide (LPS)-stimulated primary neuron, astrocyte ans microglia. Strong tPA activity was observed in cultured primary rat neuron with weaker activity in astrocyte and microglia. tPA release to extracellular space is increased with the LPS-treatment in neuron. However, the enzymatic activity of tPA was decreased mainly due to the increased expression of PAI-1 both in neuron and astrocytes but not in microglia. The expression of PAI-1 is highest in astrocytes and lowest in microglia. Actually, we were not able to identify cell-associated tPA in microglia by Western blot assay. The results from the present study suggest that astrocyte is the major source of PAI-1 in brain and plays most important role in the regulation of tPA activities by PAI-1 under inflammatory condition. The differential regulation of tPA and PAI-1 in neuron, astrocyte and microglia suggests more attention is required in the understanding of the role of local PA activity in the vicinity of individual cell types.

2815/B529

Expression Profiling of Human Bronchial Epithelial Cells after Intracellular Introduction of Small DNA Fragments.

H. Parsi[1], S. E. Hensley[2], J. B. Collins[3], R. S. Paules[3], D. C. Gruenert[1,2,4]; [1]Department of Cell Biology, CPMC Research Institute, San Francisco, CA, [2]Department of Medicine, University of Vermont, Burlington, VT, [3]National Institutes of Environmental Health Sciences, Research Triangle Park, NC, [4]Department of Laboratory Medicine, University of California, San Francisco, CA

Oligonucleotide-based therapeutic methods are emerging as a potential alternative to cDNA based gene therapy. These methods, although variable in design, all involve the introduction of exogenous nucleic acids into targeted cells. To assess the cellular response to this event, immortalized human bronchial epithelial cells were electroporated in the presence of a double stranded (ds) small DNA fragment (SDF). The expression profiles of transfected and non-transfected cells were compared at two different time points (8 and 24hr post-transfection) using an Affymetrix-type whole human genome microarray. Using the Rosetta error model, genes that were significantly up- or down-regulated post-transfection were identified. Affected genes 8 hours post-transfection were found to be included specific pathways, e.g., Notch signaling, centrosome duplication and separation, signal transduction, valine catabolism, translation initiation, apoptosis, and ubiquitin pathways. Twenty four hours post-transfection genes in the septin complex, asparagine-linked oligosaccharide (N-glycan) maturation, ATM (ataxia telangiectasia mutated) and the ATR (ATM and Rad3-related) pathways were altered. A number of these pathways are involved in cell fate and DNA repair are thus crucial to the overall efficiency of oligonucleotide therapy. This work was supported by NIH Grant DK66403.

2816/B530

Neural Crest and Glial Cell Development in Elasmobranches.

M. Juarez, M. E. de Bellard; Biology, Cal State Northridge, Northridge, CA

Neural crest cells originate from the dorsal neural tube shortly after its closure and give rise to neuronal, glial, and non-neuronal derivatives. In vertebrates, these progenitor cells also give rise to a variety of cranial and facial features, and in the lesser spotted catshark, Scyliorhinus canicula these cells have been implicated in the development of electrosensory receptors. Importantly, glial cells are responsible for myelin formation in higher vertebrates. Our work aims to identify and examine the expression of several glial proteins and transcription factors in development as well as in the formation of neural crest cells in bamboo shark (Chiloscyllium punctatum). This study is important because little is known about neural crest cell and glial development in elasmobranches. We cloned Sox8 and 9 in shark and examined the expression of Po, p75, CNPase, MBP, S100 and GFAP, in developing elasmobranches by using in situ hybridization, SEM, immunohistochemical methods and fluorescence microscopy. We observed cells that resemble neural crest which were positive for Sox8 and HNK1 in the lateral line. Seraf (a Schwann cell marker) was expressed in the hindbrain of developing elasmobranches. Finally, several areas of the developing brain showed distinct expression of myelin markers: MBP and Po. Scanning electron microscopy revealed migrating neural crest cells. This

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2817/B531

Nuclear Factor I-X Transcription Factor Is Required for Mouse Osteoblast Differentiation In Vitro.

L. A. Perez-Casellas[1, 2], C. Strohbach[1, 2], D. Strong[2, 1], T. Linkhart[2, 1]; [1]Biochemistry, Loma Linda University, Loma Linda, CA, [2]Loma Linda VAMC, Loma Linda, CA

The Nuclear Factor I (NFI) gene family plays wide reaching roles in viral DNA replication, regulation of gene transcription and development. Germline deletion studies of NFI genes have shown that deletion of *Nfi-a* causes mainly neuroanatomical defects, *Nfi-b*$^{-/-}$ mice show lung and brain defects, *Nfi-c* deletion caused primarily a disruption of tooth root and underlying mandibular bone development, and *Nfi-x*$^{-/-}$ mice have brain malformations, poor postnatal growth and develop a deformation of the spine, associated with impaired endochondral ossification and decreased mineralization in axial and peripheral skeleton. We previously reported that the Nuclear Factor I gene family was an important regulator of IGFBP-5 expression in human osteoblast-like cells. To determine the role of the four NFI genes during mouse osteoblast differentiation we determined *Nfi* mRNA expression levels in mouse bone marrow stromal cells and mouse osteoblast MC3T3-E1 cells by quantitative RT-PCR at different stages of differentiation in vitro (alpha MEM, FBS, Ascorbic Acid and 2-glycerophosphate). All four *Nfi* genes were expressed and their expression levels increased at least 10 fold during differentiation. *Nfi-x* expression was the most abundant in both differentiation models and increased the most during differentiation. To determine the role of *Nfi-x* in osteoblast differentiation, mouse *Nfi-x* expression was inhibited with specific siRNA oligonucleotides in MC3T3-E1 cells. *Nfi-x* mRNA levels were decreased at 48 hrs by 80% and mRNA levels of osteocalcin, a bone matrix protein that is highly expressed in differentiated osteoblasts, were decreased by 65% compared to non targeting control siRNA. Long term knockdown of *Nfi-x* using lentiviral short hair pin vector decreased *Nfi-x* mRNA levels more than 75% during 3 weeks in differentiation inducing conditions and strongly decreased mineralization and osteocalcin, bone sialoprotein (BSP), and Runx2 mRNA expression levels. These results suggest that NFI-X positively regulates osteocalcin, BSP and Runx2 gene expression and that NFIX has an important role in regulating osteoblast differentiation and mineralization.

2818/B532

Gene Expression Analysis on Erythroid Progenitors from Fetal Liver and Adult Bone Marrow.

L. Tai[1], M. E. Fomin[1], M. O. Muench[1,2]; [1]BSRI, Blood Systems Reseach Institute, San Francisco, CA, [2]Department of Medicine, UCSF, San Francisco, CA

The fetal liver and adult bone marrow are the major organs that support hematopoiesis in human. Particularly, erythropoiesis dominates in these tissues. Better understanding gene expression changes that occur during differentiation of committed erythroid precursors and during development is likely to find sets of genes important to the process of erythropoiesis. This study aimed at understanding the differences in genomic expression between fetal and adult erythroid development through a computational approach that integrates gene expression profiles with functional information. Gene expression analysis was performed at two stages of erythroid development. Gene expression data from twelve experiments for both subsets of fetal and adult erythroid progenitors were used to generate integrated datasets of genes associated by their biological functions. Comparisons were performed between array results from different subsets of erythroid precursors during differentiation, and between array data from fetal and adult erythroid populations. In order to understand the biological meanings of genetic profiles, genes were further annotated using a gene ontology (GO) program in their biological processes. After GO analysis, we focused on significant GO terms associated with differences in gene expression including transcription, differentiation, and biosynthesis. We found that some of these genes are increasingly expressed during development in both tissues including HMBS, and ALAS2. Furthermore, the differences in gene expression in GO terms related to transcription and differentiation were used to understand a genetic signature specific between fetal and adult erythrocyte differentiation. Differences in gene expression profiles between fetal and adult erythroid populations were built based on continuously increased or decreased gene expression with significantly different expression in fetal or adult erythroid precursors. The expression of selected genes was confirmed by qPCR. Our data demonstrated differences in transcriptional and differential profiles between fetal and adult erythroid progenitors during erythropoiesis.

2819/B533

Identification and Characterization of a Novel Lamin A/C (LMNA) Interacting Protein (LIP) That Binds DNA Regulatory Regions of Developmental and Cell Cycle Genes.

P. Burgon[1,2], E. Ahmady[1,2], B. Vulesevic[1], L. Kenney[1]; [1]University of Ottawa Heart Institute, Ottawa, ON, Canada, [2]Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON, Canada

Lamin A/C is expressed in all tissues of the body, however specific mutations in the LMNA gene result in discrete tissue specific phenotypes, such as muscular dystrophy. This suggests that the lamin A/C protein in skeletal muscle interacts with specific muscle factor(s) and that modification of these interactions are responsible for the pathogenesis of skeletal muscle based laminopathies. Using a yeast two-hybrid screen, we identified a novel protein that interacts with Lamin A/C (lamin interacting protein, LIP). Exhaustive bioinformatics analyses of LIP reveal it to be a novel single copy gene that is conserved in Amniota that encodes proteins with no known structural or functional domains. LIP protein is primarily expressed in brain, cardiac and skeletal muscle. **Objective:** To define the functional role of LIP in muscle. **Results:** LIP is expressed endogenously in a mouse myoblast cell line (C2C12) and is co-localized to the nuclear envelope and PML bodies of the nucleus. Initiation of C2C12 differentiation led to a 3-fold increase in LIP expression peaking at 24hrs and LIP continued to be expressed in differentiated myotubes. Specific knockdown of LIP in C2C12 cells by shRNAi led to significant ($p<0.01$) reduction in Pax7 expression with a concurrent reduction in Myf5, MyoD & myogenin and subsequent inhibition of myotube formation. Whereas over-expression of LIP led to 10-fold increase in Pax7 expression with a subsequent >20-fold increase in Myf5, MyoD & myogenin expression. To determine whether LIP's modulation of gene expression is directly through chromatin binding a ChIP study was performed with a LIP specific antibody against undifferentiated and differentiated C2C12 cells. Several 5' proximal regions of developmentally important genes (notch 2 and GATA6) as well as several other genes implicated in cell cycle control (nek7) have been identified. Current experiments are

elucidating the regulatory role LIP on specific expression profiles. **Conclusion:** LIP is a novel LMNA interacting protein that regulates a number of developmentally and cell cycle important genes and may be critical for stem or satellite cell based maintenance, repair and regeneration of tissues and organs. **Funding** CIHR to PGB

2820/B534

Runx2 and Bmp2 Are Insufficient to Induce Mineralization of Mesenchymal NIH3T3 Cells but Are Associated with Elevated Expression of Bone Marker Genes.

A. Stephens, N. Morrison; Griffith University, Southport, QLD, Australia

Runx2 and Bmp2 are important regulatory factors involved in osteoblast differentiation and function. Here we investigated whether Runx2 and Bmp2 could promote the trans-differentiation of mesenchymal NIH3T3 fibroblasts into mineralizing osteoblast-like cells. Furthermore, we performed quantitative gene expression analysis to provide an insight into genes regulated by Runx2 and Bmp2. Stably transfected NIH3T3 cell lines overexpressing Runx2 were generated and recombinant Bmp2 was used to supplement the culture media at 200 ng/ml. Alizarin Red S staining was used to assess extra cellular matrix mineralization and neither Runx2 nor Bmp2 were able to induce bone nodule formation. The treatment of cells with a combination of both factors also failed to produce a mineralized matrix. We next investigated alkaline phosphatase; an enzyme highly expressed in osteoblasts. Enzyme activity levels were measured after 3, 5, 9 and 12 days of culture in osteogenic media. The assay revealed there were no significant differences in alkaline phosphatase activities in response to Runx2 or Bmp2 treatment alone or in combination at any time point confirming the factors were not sufficient to promote an osteoblastic phenotype. Cellular proliferation was assessed using the MTT method and the assay demonstrated that Runx2 overexpressing cells had a highly significant growth advantage outgrowing control and Bmp2 treated cells 1.7 fold ($p = 6.64 \times 10\text{-}5$) after four days in culture. Bmp2 treatment did not significantly alter cell proliferation (1.05 fold, $p = 0.54$). We further evaluated the effects of Runx2 and Bmp2 by investigating gene expression. Runx2 was able to significantly induce the bone markers Osterix (74 fold), MMP13 (566 fold), Alkaline phosphatase (6 fold) and Osteocalcin (15 fold). Bmp2 did not overtly influence gene expression on its own however when combined with Runx2 was able to further induce Osterix (1.6 fold) and alkaline phosphatase (2.8 fold) over Runx2 alone indicating synergism between the two factors. Our data shows that Runx2 and Bmp2 are insufficient to induce bone nodule formation in NIH3T3 cells however are capable of inducing bone maker gene expression.

2821/B535

Dynamic Regulation of the Tissue-Specific Transcription Factor CeTwist.

S. G. Meyers, A. K. Corsi; Biology, the Catholic University of America, Washington, DC

The *hlh-8* gene encodes a basic helix-loop-helix transcription factor called Twist that is involved in mesoderm development and the morphogenesis of enteric and sex muscles in *C. elegans*. Twist binds to the canonical binding site CANNTG referred to as an E-box. The *hlh-8* gene is composed of 5 exons with a 2 kb intron after the first exon. Because the function of *hlh-8* is dependent on the gene being expressed in the appropriate cell type, it is important to understand how the expression of the *hlh-8* gene is regulated. Typically, elements in the promoter of a gene determine the regulation of gene expression in different tissues. However, only elements that control expression in a subset of Twist containing tissues have been discovered in the promoter. Therefore, additional elements must exist elsewhere and a hypothesis was developed that regulatory elements are present in the large first intron. We are exploring the *hlh-8* intron using a construct that contains a basal promoter and can be activated to express GFP in a variety of tissues by juxtaposition to a tissue-specific enhancer. Constructs containing portions of intron 1 have revealed two regions, with a single E-box each, which are sufficient to drive expression of *gfp* in a subset of tissues that express Twist. Furthermore, expression of *gfp* is lost when both E-boxes are disrupted. Our hypothesis is that these E-boxes are important for *hlh-8* autoregulation. Results from expressing these reporters in *hlh-8* null mutants and examining Twist binding to the E-boxes by *in vitro* gel shift analysis support this hypothesis. Additionally, we are also currently characterizing a mutant with a large deletion (646 bp) in the first intron of *hlh-8*. Altogether, our results will lead us to an understanding of tissue-specific regulation of *hlh-8*. Intron elements appear to control expression in differentiated tissues, whereas it has been shown previously, that other factors regulate expression in undifferentiated cells. Intriguingly, we have also observed that different Twist containing dimers function in undifferentiated versus differentiated tissues and the distinct gene regulation may influence tissue-specific dimer formation.

2822/B536

Transcription of the Osteogenic Runx2 Gene Is Co-Stimulated by Sp1 and Ets/Elk at a Polymorphic Bipartite Promoter Element (Y-Repeat).

Y. Zhang, M. Hassan, R. Xie, J. Stein, J. Lian, A. van Wijnen, G. Stein; Cell Biology, Umass Medical School, Worcester, MA

Transcriptional control of Runx2 gene expression through two alternative promoters (P1 and P2) is critical for the execution of its function as an osteogenic cell fate determining factor. In all vertebrates examined to date, the P1 promoter contains a purine-rich region (-303 to -124) that separates two regulatory domains. The length of this region differs dramatically between species even within the same order. Using deletion analysis, we show that part of this purine-rich region (-200 to -128) positively regulates Runx2 P1 transcription. The -200/-128 region contains a duplicated element (Y repeat) and electrophoretic mobility assays and chromatin immunoprecipitations reveal that this element binds at least two different classes of DNA binding factors related to GC box binding proteins (e.g., Sp1) and Ets-like factors (e.g., Ets1 and Elk1). Forced expression of Sp1 increases Runx2 P1 promoter activity through the Y repeats and siRNA depletion of Sp1 decreases Runx2 gene expression. Similarly, exogenous expression of wild type Elk1, but not a transcriptionally defective mutant, enhances Runx2 gene expression. Sp1 is most abundant in proliferating cells and Elk1 in post-confluent cells; both proteins are transiently co-expressed during MC3T3 osteoblast differentiation when Runx2 expression is enhanced. Taken together, our data suggest that basal Runx2 gene transcription is regulated by synergistic interactions between Sp1 and Ets-like factors to control Runx2 expression during osteogenesis.

2823/B537

Determining the Flanking Regions of Insertions in Arabidopsis Thaliana Enhancer Trap Lines.

J. Diaz, M. Zavala; Biology Department, California State University Northridge, Northridge, CA

Arabidopsis thaliana is a small flowering plant used as a model organism for plant biology. The Arabidopsis offers important advantages to molecular biology because it has a small genome that was sequenced in the year 2000. It is easy and quick to grow, easy to transform using Agrobacterium tumefaciens, and has a rapid life cycle that takes 6 weeks from germination to mature seed. We are looking for the location of the insertions of Gal4 -GFP that result in root specific enhancer trap lines of Arabidopsis thaliana. TAIL-PCR (thermal asymmetric interlaced polymerase chain reaction, Liu 1995), is the method we are using to determine the flanking regions of where the trap lines are located in the genome. We are probing Arabidopsis J571 and J481 genomes as templates. We have modified the previously stated method, which has been used on different strains of mutated Arabidopsis with varying success. Our ultimate goal is to determine the flanking regions around the inserts.

2824/B538

Deletion of H11 Kinase Accelerates Heart Failure in the Overloaded Heart.

H. Qiu, C. Hong, S. Gao, D. Vatner, S. F. Vatner, C. Depre; Cell Biology and Molecular Medicine, UMDNJ, New Jersey Medical School, Newark, NJ

H11 kinase (H11K), a heat shock protein expressed mainly in the heart, is up-regulated upon pressure overload, and cardiac-specific over-expression of H11K in a transgenic model induces cardiac hypertrophy. We tested in an H11K knockout (KO) mouse the hypothesis that H11K deletion would accelerate the transition into heart failure (HF) in a context of pressure overload induced by aortic banding. In basal conditions (4/group), no differences were found between wild type (WT) and KO mice in terms of left ventricular (LV) mass and contractility (LV ejection fraction: 70±1 vs 69±3%). After two weeks aortic banding (6/group), the KO mice began to show signs of heart failure as LV ejection fraction fell to 45±3%, $P<0.05$ greater than the fall in LVEF in WT (62±5%). Lung weight/tibial length, another sign of heart failure, rose from 7.0±0.5 to 16.2±2.5 in KO mice after banding, $P<0.05$, whereas it did not increase in WT. LV end-diastolic pressure also rose more in KO, from 2.5±2.0 to 22±4 mmHg after banding, $P<0.05$ compared with WT (7±4 mmHg). Stimulation of cardiac cell growth after banding was comparable between WT and KO (myocyte cross-sectional area [μm^2]: 278±5 and 328±46 in sham WT and KO, NS; 494±46 and 457±15 in WT and KO after banding, NS; both, $P<0.05$ vs sham). Similarly, the sensitivity to apoptosis was not affected in KO compared to WT (TUNEL-positive cells per μm^2: 0.7±0.1 and 0.8±0.1 in sham WT and KO, NS; 1.3±0.2 and 1.4±0.2 in WT and KO after banding, NS; both, $P<0.05$ vs sham). However, KO mice were characterized by more accumulation of collagen upon aortic banding (% surface covered by collagen: 3.7±0.9 and 3.2±0.4 in sham WT and KO, NS; 12.5±2.2 and 22±1.7 in WT and KO after banding, $P<0.01$). Therefore, H11K is a stress-responsive chaperone in the heart, as its deletion does not affect basal cardiac function but it precipitates the transition into HF during overload, which could be due to increased collagen accumulation.

Invertebrate Development (2825 – 2833)

2825/B541

THO Complex Is Required for the Spermatogenesis in Drosophila.

H. Kim, S. Moon, Y. Chung; University of Seoul, Seoul, South Korea

THO complex is highly conserved from yeast to human as a multi-subunit protein complex that is known to play a key role in cotranscriptional mRNA processing and nuclear export in eukaryotic cells. In yeast cells, the majority of mRNAs requires the THO complex for their transcription and nuclear export, however unexpectedly only subset of mRNAs appears to be dependent on the complex for their nuclear export in metazoan cells. Nevertheless, its specific character in vivo has been poorly understood. Recently, we isolated a novel Drosophila male sterile mutant, garmcho. Positional cloning indicated that garmcho encodes a component of Drosophila THO complex, THOC5. garmcho shows male sterility as well as disrupted morphology of nucleoli in primary spermatocytes. In addition, fusion protein GFP::THOC5 reveals its unique pattern as a single dot near nucleolus in early stage of primary spermatocytes and as a ring shape around the nucleolus in late stage of primary spermatocytes. Since Drosophila testis-specific TAFs (tTAFs) are known to express genes required for meiotic entry and terminal differentiation, we considered a possible interaction of THO complex with tTAFs. By using immunohistochemisty or GFP-fusion protein, we found that not only were tTAFs severely mislocalized in garmcho testes, but the localization of GFP::THOC5 was also disruptive in tTAF mutant testes. When analyzing distribution of don juan mRNA, a target mRNA of tTAFs, we found its distribution in nucleus is abnormal, while nuclear export looks normal. Taken together, our data suggested the specific role of THO complex in spermatogenesis probably by organizing nuclear integrity.

2826/B542

No Poles* Encodes a Predicted E3 Ubiquitin Ligase Required for Early Embryonic Development of *Drosophila.

J. Merkle[1], J. Rickmyre[1], A. Garg[2], E. Loggins[1], J. Jodoin[1], E. Lee[1], L. Wu[2], L. A. Lee[1]; [1]Cell and Developmental Biology, Vanderbilt University Medical School, Nashville, TN, [2]Center for Biosystems Research, University of Maryland Biotechnology Institute, College Park, MD

In a screen for cell-cycle regulators, we identified a *Drosophila* maternal effect-lethal mutant that we named "*no poles*" (*nopo*). Embryos from *nopo* females undergo mitotic arrest with barrel-shaped, acentrosomal spindles and misaligned chromosomes during the rapid S-M cycles of syncytial embryogenesis. *nopo* encodes a candidate RING domain-containing E3 ubiquitin ligase and in vitro studies by others have shown that the mammalian homolog of NOPO (TRIP) exhibits E3 ligase activity. TRIP was originally identified as an interacting partner of tumor necrosis factor (TNF) receptor-associated factor (TRAF) and recently shown to be required for early embryonic development in mice. Our genetic studies show that Checkpoint kinase 2 (Chk2) is activated in *nopo* mutants, suggesting that *nopo* plays a role in maintenance of genomic integrity. We find that *nopo*-derived embryos exhibit shorter interphases during cycle 11. Our data suggest that truncation of

interphases during S-M cycles in *nopo*-derived embryos results in mitotic entry prior to completion of DNA replication, eliciting Chk2-mediated mitotic arrest and embryonic lethality. Our phenotypic analyses and yeast two-hybrid results indicate that NOPO and BEN/UEV1A, a heterodimeric E2 conjugating enzyme (Ubc13/Uev1A homologs), form an E2-E3 ubiquitination complex required for genomic stability during embryogenesis. Current efforts are focused on identifying NOPO targets and interactors so as to elucidate the mechanism by which NOPO promotes genomic stability during *Drosophila* development. We are also using mammalian cultured cells to assess the biological roles of human TRIP. We find that TRIP localizes to nuclear puncta, and we are performing knock-down experiments to determine if human TRIP plays an evolutionarily conserved role in maintaining genomic integrity.

2827/B543

Regulation of Twinstar (Drosophila Cofilin) Phosphorylation by LIMK1 and Slingshot Is Required for Drosophila Ventral Furrow Morphogenesis.

A. Goyal, M. Witzberger, S. Dowd, J. Minden; Carnegie Mellon University, Pittsburgh, PA

Ventral furrow formation, the first morphogenetic event during Drosophila gastrulation, is driven by coordinated cell shape changes. In this process, columnar-shaped, ventral cells are converted into wedge-shaped cells, which invaginate to form the mesoderm. Ventral furrow formation is initiated by the activation of the Toll receptor, and the subsequent expression of the transcriptional regulators, Twist and Snail. It is not known how these transcriptional regulators bring about the cytoskeletal changes that underlie the cell shape changes necessary to drive ventral furrow formation. In our previously conducted proteomic screen to discover ventral-specific, protein changes during ventral furrow morphogenesis, the most prominent cytoskeleton-associated protein found to be altered was Twinstar, Drosophila cofilin. Here we show that non-phosphorylated (activated) Twinstar is enriched in ventral cells, and that it is maintained at this level by the combined effect of increasing Twinstar phosphatase, Slingshot, and decreasing Twinstar kinase, LIM kinase 1. Treatments that increase the level of phosphorylated (inactive) Twinstar, such as decreasing Slingshot abundance or increasing LIM kinase 1 abundance, interfere with normal ventral furrow formation. We further show that Slingshot is enriched in ventral cells under the transcriptional control of Twist, and that LIM kinase 1 is specifically lost in ventral cells during furrow formation. Together, our findings demonstrate that Toll-dependent regulation of Twinstar plays an important role in the cell shape changes associated with ventral furrow morphogenesis.

2828/B544

Non-Equilibrium Computer Simulations of Morphogenesis Show That Elevated Basolateral Contractility Is Essential for Ascidian Endoderm Invagination.

K. Sherrard, E. Munro; Biology, University of Washington, Friday Harbor, WA

To characterize the cellular and mechanical basis of ascidian endoderm invagination, we have amassed detailed experimental data and simulated how cortical contractility could drive this morphogenesis. Our experimental evidence suggests the invagination occurs in two steps: (1) apical contraction forms a placode and (2) basolateral contraction shortens the endoderm causing it to sink inwards. Using a mechanically precise computational simulation of a contractile and visco-elastic cortex on a 2D ascidian embryo cross-section, we tested this hypothesis, exploring which combinations of localized cortical contractility generated placodes and invaginations. Wide-ranging searches of tension parameter space largely confirmed our hypothesized invagination mechanism: most placodes formed with conditions of strong apical contraction, and no invaginations occurred without elevated basolateral contractiliity on the invaginating endoderm cells. Interestingly, either step could occur with negative (ectoderm-dominated) apical contraction. In step 1, elevated basolateral contractility of the ectoderm caused these columnar cells to round, compressing the endoderm and compensating for a lack of apical contraction; the ectoderm rounds and spreads laterally in the real embryos at this stage in a form of epiboly. In step 2, temporary invaginations could form given sufficiently basolateral contractility on the endoderm, which would later regress if either endoderm apical contraction or ectoderm basolateral contraction was insufficient; in the real embryos the mechanism preventing regression appears to be circumapical contractility on the endoderm. This morphogenetic simulation departs from most others in representing viscous as well as elastic cortical properties, accurately representing relaxation of the cortex over tens of seconds due to the continued assembly and disassembly of the actomyosin network. Thus not just the endpoint but the kinematics of simulations through time can be analyzed. Crucially, many additional solutions are possible that do not depend on elastic equilibrium but are nevertheless sufficiently stable to be characteristic of real embryos.

2829/B545

Force Anisotropy during Germ Band Retraction in *Drosophila* Embryos.

H. E. Lynch[1], B. Rosenthal[3], M. S. Hutson[1,2]; [1]Physics & Astronomy, Vanderbilt University, Nashville, TN, [2]Biological Sciences, Vanderbilt University, Nashville, TN, [3]Physics, Duke University, Durham, NC

One of the four major stages of *Drosophila* embryogenesis is germ band retraction. This stage involves the movement of two epithelial sheets: the germ band and the amnioserosa. These movements are driven by forces within the epithelial sheets; however, these forces have not yet been identified. To elucidate the forces acting in the germ band itself, we made 15-micron laser incisions in each of its twelve segments. These incisions were made in two directions: parallel and perpendicular to the segment boundaries. The resulting wounds were then compared over time. All wounds initially recoiled and reached a maximum area. Most then went through a healing process. The forces in the germ band can be qualitatively identified by the amount and direction of retraction in the two incision orientations. For segments A5-A7, parallel and perpendicular wounds expand to approximately the same maximum area; however, these wounds differ in aspect ratio. All wounds expanded more in the direction parallel to the segment boundaries. Thus, parallel wounds expanded to become longer, but thinner, than perpendicular wounds. These results imply an underlying force anisotropy. This anisotropy is more consistent with models in which retraction of the germ band is driven by externally applied forces, e.g. from the amnioserosa, as opposed to autonomous forces generated in the germ band.

2830/B546

Examining a Role for Glia during Development of the Abdominal Nervous System in Drosophila.

J. Fernandes, R. Rachamduggu, S. Banerjee, M. Dorr, B. Kathleen, M. Seiffert, K. Hardin; Zoology, Miami University, Oxford, OH

During metamorphosis in holometabolous insects, most tissues are dramatically restructured. In Drosophila, the muscle system is completely re-organized to accommodate adult specific behaviors such as flight. The nervous system is also restructured: while many neurons are eliminated, others persist to innervate new adult muscle targets, and many interneurons are generated anew to be integrated into new neural circuits. In addition to the expansion of the thoracic ganglion, which is the seat of locomotor control, another striking change is seen in the abdominal ganglion. It becomes reduced in size and of the 8 pairs (A1-A8) of larval abdominal nerves, only A1-A3 are present as distinct pairs, while the remaining 5 pairs (A4-A8) fasciculate to form a terminal nerve trunk. We have followed the remodeling during pupal stages using nervous system specific markers and show that the transition in shape occurs just after one day of metamorphosis (30% of pupal development). We show that transition occurs just as muscle fiber formation is beginning in the periphery. We also show that an extensive glial remodeling occurs during this time as evaluated by anti-repo staining. Future studies will be aimed at disrupting glia to examine how they might facilitate formation of the terminal nerve trunk. Glia are known to insulate nerves a feature important for physiological function, and we are interested in exploring a developmental role during the restructuring of peripheral nerves in Drosophila.

2831/B547

Development of the Larval Muscle System in the Mussel Mytilus Trossulus (Mollusca, Bivalvia).

V. Dyachuk, N. Odintsova; Biochemistry and Biophysics, Zhirmunsky Institute of Marine Biology, Vladivostok, Russian Federation

The aim of this study was to reveal temporal expression pattern of muscle proteins during mussel ontogenesis, and to perform morpho-functional characterization of developing muscles. We revealed the expression patterns of myosin, paramyosin, myorod and twitchin during mussel ontogenesis. We showed that paramyosin can not be considered as a marker of larval muscles, since it was detected in actomyosin extracts of all the developmental stages including unfertilized eggs. Myorod and myosin were expressed at blastula and trochophore stage, respectively. Expression of twitchin starts at the blastula stage, simultaneously with myorod. The protein composition of larva and adult muscles is different: paramyosin is much less abundant in larval extracts, while relative amount of actin and myosin does not change significantly during mussel development. Such low content of paramyosin and almost undetectable amount of myorod indicates that unlike in adult mussel larva musculature consists mostly of striated fibers. We found that frequency of spontaneous contractions of veliger retractors is about 30 times higher than one of the adult smooth muscle and corresponds to the frequency of fast contraction of invertebrate muscles. We monitor remodeling of larva musculature using CLSM. We demonstrated the assembly of nascent myofibrils at the early trochophore stage. Later, well defined striated musculature was observed and these striated muscles are completely resorbed during larval metamorphosis. Once smooth muscle cells appear in veliger shell adductor, they are present in all the developmental stages and finally become the exclusive muscle type in adults. We also demonstrated striated pattern of myosin filaments in the mussel larval muscle by the second harmonic generation imaging. SHG imaging detects myosin assembled into a contractile lattice only, but due to non-linear nature of the second harmonic generation this imaging modality is suitable to acquire high-resolution three-dimensional stacks. These results provide the first strong evidence that mussel larva metamorphosis is accompanied by massive degradation of striated muscles and development of smooth muscle tissue capable of catch-contraction.

2832/B548

Characterization of Acid Phosphatase Activity during Flight Muscle Histolysis in the House Cricket, Acheta Domesticus.

A. N. Albury[1,2], R. Oliver[2], T. A. Mousseau[1]; [1]Biological Sciences, University of South Carolina, Columbia, SC, [2]Biology, Chemistry and Environmental Sciences, Benedict College, Columbia, SC

Acid phosphatase (AcP) activity was characterized in the dorsal longitudinal muscles of the house cricket during histolysis. AcP is recognized as a biochemical marker of lytic activity and is an indicator of cellular autolysis. Muscle homogenates from crickets ranging from Day 0 - 3, including two experimental treatments—Day 3L (cervically ligated to prevent histolysis) and 3LM (ligated crickets with hormone replacement) were assayed for total protein and AcP activity. Levels of AcP activity were altered during the course of histolysis. AcP activity was highest on Day 3 (0.700 ± 0.021 pmols/min; mean ± SE) and was significantly greater than all other treatments ($p<0.0001$; n=4; Tukey). This coincided with histolysis, which is marked by the dramatic loss of protein in the flight muscle at Day 3 (1.636 ± 0.037 mg/paired DLMs). AcP activity levels at Day 3L (0.214 ± 0.0084 pmols/min) were ≈ 69% less than that of Day 3 ($p<0.0001$; n=4; Tukey) and did not differ significantly from Day 1 ($p=0.9723$; n=4; Tukey); hence, ligation prevents histolysis and reduces AcP activity. Hormone replacement with methoprene (Day 3LM—0.474 ± 0.0184 pmols/min) restored histolysis and elevated AcP activity. Sodium tartrate was used as an inhibitor of AcP activity and was shown to reduce AcP activity by 18 - 62%. AcP activity at Day 3 (0.264 ± 0.0076 pmols/min) was reduced compared to the control by ≈ 62% ($p<0.0001$; n=4; Tukey), suggesting elevated levels of tartrate-sensitive AcP. AcP activity at Day 4, 3LM and 2 were also reduced. The AcP activity at Day 0, 3L and 1 were not significantly affected by sodium tartrate. Muscle homogenates were analyzed in a Native PAGE using a modified Gomori stain with p-nitrophenylphosphate and lead acetate. The gel revealed two bands with AcP activity; these bands were excised and reanalyzed on a 10% SDS-PAGE showing a distinctive band at 147-kDa. Electron microscopy and histochemistry for AcP localization showed lysosomal and autophagic vesicles engulfing cellular organelles. These results support an active role of AcP in flight muscle histolysis and autophagy of organelles.

2833/B549

Cloning and Analysis of *S. Purpuratus* Argonaute Proteins.

L. Chuang, S. A. Seipel, E. M. Bonder; Department of Biological Sciences, Rutgers, the State University of New Jersey, Newark, NJ

In previous work, Rodriguez et al. (2005) identified and characterized seawi, the *S. purpuratus* orthologue of Piwi, opening the possibility that the argonaute family of proteins play essential roles in the establishment of the developmental axis and the specification of stem cell niches in the sea urchin system. Recently, Song and Wessel (2007) used sea urchin genome information to deduce the existence of Ago1, Ago2, and seawi-like argonaute family members. In this report, we provide the characterization of expressed transcripts for seali, Ago1, and

Ago2. PCR amplification of cDNA prepared from unfertilized egg mRNA followed by nucleic acid sequencing resulted in the identification of the entire coding sequences for seali (EU334659), Ago1 (EU733246), and approximately two-thirds of Ago2. Each of the transcripts is expressed during the first 75 hours of development and in adult testis. As anticipated, the PIWI domain of all expressed argonautes contain the conserved aspartic acid and histidine residues required for potential slicer activity. Additionally, the PIWI domains contained the requisite sequences for anchoring the 5' end of small RNAs. Comparison of sequences present along the RNA binding pocket of PAZ domains identified amino acid residue differences between the argonaute and seawi subgroups that may reflect differences in small RNA selection and/or cellular function. Thus, sea urchin embryos express a full complement of argonautes capable of utilizing si-, mi-, and piRNA mediated mechanisms for regulation of protein expression.

Cell Polarity II (2834 – 2856)

2834/B550

NMDA Receptor Signaling Is Required for Correct Spindle Orientation and Protein Localization during the Early Asymmetric Cleavages.

A. Nakamoto[1], J. R. Cooley[1,2], D. Steadman[1], L. M. Nagy[1]; [1]Molecular and Cellular Biology, University of Arizona, Tucson, AZ, [2]Biology, University College London, London, United Kingdom

Asymmetric cleavages and stem cell divisions are essential for generating cellular diversity during development. Asymmetry begins with the polarization of the mother cell along a specified axis. The mitotic spindle aligns with this polarity and cell fate determinants are distributed asymmetrically along the division axis segregating to one of the daughter cells. Here we report that N-methyl-D-aspartate (NMDA) receptor signaling is required for correct spindle orientation and protein localization during the stereotyped asymmetric cell divisions in an early mollusc embryo. The presence of the phosphorylated form of the NMDA receptor subunit (NR-1) was identified via an antibody-based proteomics approach. The phosphorylated NR-1 antigen is expressed on the cell membrane and peri-nuclear region in the early cleavage stages and undergoes dynamic changes in intracellular location within a cell cycle. To assay the role of NMDA receptor signaling, we applied a highly selective noncompetitive NMDA receptor antagonist (MK801) to block the input of the receptor during the divisions between 4-cells and 12-cells. We found that the typical asymmetric cleavages that generate the specific arrangement of small daughter cells are lost. The spindles of the mother cells align nearly perpendicular to the normal orientation, and they divide abnormally into two blastomeres of equal size. Using the antigen(s) recognized by phosphorylated PKC-pan (pPKC-pan) antibody as a marker, we examined whether NMDA receptor signaling is also required for asymmetric protein localization. In normal development and control treatments, pPKC-pan antigen(s) always localizes to the apical cortex of the mother cells and subsequently segregates to the smaller daughter cells. In contrast, when NMDA receptor signaling was blocked with MK801, pPKC-pan antigen(s) localized on ectopic cortical sites of the mother cells. In some cases, the antigen(s) localized on the cortex near the abnormal lateral spindle attachment site. These results suggest a novel role for NMDA signaling in the determination of spindle orientation and cortical polarity in early asymmetric cleavages.

2835/B551

Dissecting Wnt's Role in Cell Polarization by Applying Purified Wnt Signals to Single Cells.

M. Roh[1], A. Mikels[2], R. Nusse[2], B. Goldstein[1]; [1]University of North Carolina - Chapel Hill, Chapel Hill, NC, [2]Stanford University, Stanford, CA

Cell polarization often involves intracellular motility orchestrated by cell-cell signalling, a phenomenon that remains under-explored in developmental systems. We are studying the four-cell stage Caenorhabditis elegans embryo, a system where genetics and cell manipulation approaches can be combined to dissect mechanisms of cell polarization by extracellular signals. At this stage, two signals from one cell are required to orient the mitotic spindle in a neighbouring cell. These signals are MOM-2 (a Wnt family protein) and MES-1 (a transmembrane protein). Despite the importance of Wnt signaling in development and cancer biology, it is not clear in any system whether Wnt proteins can act as direct, instructive cues (provide positional information) during the establishment of cell polarity. Cell manipulation experiments suggest that a MOM-2/Wnt-expressing cell can act as an instructive cue during polarization, but whether this is a direct effect of MOM-2/Wnt or of a downstream player is not known. We have developed methods to present purified MOM-2/Wnt on beads to single responding cells. We have found that although cells expressing MOM-2/Wnt act as instructive cues, purified MOM-2/Wnt coated beads are not instructive, nor inactive, but behave instead as classic permissive signals. This result suggests that in certain contexts, Wnt protein is required for cell polarization toward other signals. To further understand Wnt's role in cell polarization, we are dissecting the mechanisms by which four key, downstream proteins become asymmetrically localized. For example, LET-99 is a cortical DEP-containing protein that localizes near all cell-cell contacts and then becomes excluded from the site of signalling. We have found that depletion of LET-99 from the signalling site occurs quickly; just minutes after cell contact, and FRAP experiments suggest that it is relocalized by a diffusion trapping mechanism.

2836/B552

Dynamin Regulates Actin Cytoskeleton Remodeling for Metaphase Furrow Formation through Diaphanous in the Syncytial *Drosophila* Blastoderm.

R. Rikhy[1], M. Mavrakis[2], J. Lippincott-Schwartz[1]; [1]Cell Biology and Metabolism Program, NICHD, NIH, Bethesda, MD, [2]Laboratoire de Génétique et de Physiologie du Développement, Institut de Biologie du Développement de Marseille (IBDM), Marseille, France

Dynamin is a high molecular weight GTPase that has been well studied for its role in vesicle fission at the plasma membrane during endocytosis. It links fission events with cytoskeletal remodeling by recruiting actin-binding proteins such as cortactin. The syncytial *Drosophila* embryo is widely used to study the molecular mechanisms of membrane trafficking coupled with cytoskeletal rearrangements. The first 13 nuclear divisions occur with incomplete cytokinesis and the plasma membrane extends around each nucleus to form individual epithelial cells during the interphase stage of the 14th division. The epithelium like plasma membrane is organised to form buds that incompletely surround the nuclei. Actin present in caps in interphase is disassembled to form rings in metaphase. These promote membrane

invaginations generating metaphase furrows that separate adjacent spindles. Our studies have focused on determining whether dynamin has a role in regulating actin cytoskeleton remodeling during metaphase furrow formation. Temperature sensitive mutants at the single *Drosophila* dynamin locus, *shibire* showed a loss of metaphase furrow formation at restrictive temperatures. Both the plasma membrane and actin rings are missing at metaphase resulting in an increased incidence of tripolar spindles. An enhanced accumulation of dynamin and clathrin on the membrane at restrictive temperatures correlates with the known block in endocytosis. There is a decrease in localization of dynamin binding partners such as cortactin. Diaphanous, a formin known to be essential for metaphase furrow formation, is not localized to the plasma membrane. Both *shibire* and *diaphanous* mutants also show a loss of anillin and septin localization. Further, the polarized distribution of diaphanous and patj is severely affected. These combined data show that dynamin plays an important role in regulation of polarized distribution and localization of cortactin, patj, diaphanous, anillin and septin for metaphase furrow formation.

2837/B553

Regulation of Asymmetric Cell Division by Modular Allostery in the MAGUK Discs-large.

J. M. Jacobson[1,2], R. Newman[2], K. Prehoda[1,2]; [1]Chemistry, University of Oregon, Eugene, OR, [2]Institute of Molecular Biology, University of Oregon, Eugene, OR

Membrane Associated Guanylate Kinase-like (MAGUK) proteins organize signaling and cytoskeletal proteins at sites of cell-cell contact. An intramolecular interaction between the MAGUK SH3 and GK domains is thought to be important for regulated complex assembly, but the mechanism by which regulation occurs is unclear. In this work, we examined both the significance of the intramolecular interaction and the mechanism of regulated complex assembly. The Drosophila MAGUK Discs-large (Dlg) is required during development for asymmetric cell division of central nervous system neuroblasts, and in the adult fly for organization of neuromuscular junction proteins. We examined whether the dlgsw allele, which encodes a form of the protein that disrupts the SH3-GK intramolecular interaction, but leaves the individual domains intact, can regulate neuroblast asymmetry. We find that, while Dlgsw protein localizes properly, it is completely non-functional, indicating that SH3-GK interaction plays a critical role in Dlg function. To identify the functional mechanism by which the SH3 and GK interact, we screened for mutations that overcome the regulated complex assembly that the interaction mediates. We identified numerous mutations that allow for binding of the SH3-GK to the protein GukHolder (GuKH), which is normally inhibited. The location of these mutations indicate that the region between the SH3 and GK, termed the "HOOK", plays a critical role in regulating complex assembly. Furthermore, mutations in the GK domain, which adopts a kinase bi-lobed structure, suggest that allostery in this domain is also important for controlling ligand binding. We conclude that regulated complex assembly by the MAGUK SH3-GK module is an essential part of its function and that it occurs through a mechanism that is more sophisticated than previously appreciated.

2838/B554

The Function of Nak (Numb Associated Kinase) in Protein Trafficking in Drosophila Salivary Gland Cells.

Y. Peng, W. Yang, T. Lai, W. Lin, C. Chien; Institute of Molecular Biology, Taipei, Taiwan

Protein trafficking is a complicated process in polarized cells. It is important to establish or maintain specific functions of the cells. We found Nak is associated with AP-1 and AP-2 complex, which are required in endocytosis and protein trafficking in the cells. To understand the function of Nak in protein trafficking, we choose Drosophila 3rd instar larva salivary gland as the model system. The salivary gland cell size is increased due to the abundant protein synthesis. The plasma membrane area also needs to be increased in accompany. During this maturation process, we found that Dlg and aPKC in WT cells would be transited from the apical side to the basal side. In nak mutants, the growth of salivary gland cells and the transition of Dlg and aPKC are defected. Dlg is kept enriched in the apical side, or ectopically distributed in the cytoplasm. The distribution of the apical stationary protein, Syx1A, is normal in nak mutants. These data indicates that Nak specifically regulates the distribution of some basalized proteins. In AP-1 and AP-2 complex mutants, the transition of Dlg is also defected. Dlg is kept enriched in the apical side in both mutants, or ectopically distributed in the cytoplasm and some cellular compartments in AP-1 complex mutants. Therefore, we propose the function of Nak on the recruitment or activation of AP-1 and/or AP-2 complexes, which are required for the establishment or maintenance of the basalized proteins through endocytosis and recycling routes in the polarized cells.

2839/B555

The Antagonistic Relationship between Basolateral Polarity Proteins and Crumbs Regulates Apical Basal Polarity and Tracheal Morphogenesis.

P. Laprise[1], U. Tepass[2]; [1]Medical Biology, Laval University, Québec, QC, Canada, [2]Cell and Systems Biology, University of Toronto, Toronto, ON, Canada

The formation and dynamic maintenance of polarized epithelial tissues is crucial for embryonic development and physiology of adult organs. Recent work showed that establishment of epithelial apical basal polarity requires the competitive activity of apical and basolateral protein complexes. However, the full complement and the function of polarity regulators remain an area of intense investigation. Here we describe a novel group of epithelial polarity proteins that is composed of the FERM domain proteins Yurt (Yrt) and Coracle/Protein 4.1 (Cora) and the transmembrane proteins Na+/K+-ATPase and Neurexin IV (Nrx-IV). This 'Yrt/Cora group' maintains basolateral membrane stability of epithelial cells and shows negative regulatory interactions with the apical determinant Crumbs (Crb). Simultaneous loss of Yrt and Cora causes a dramatic apicalization phenotype that is much stronger than individual null phenotypes, indicating that these FERM proteins have overlapping functions. Further genetic analysis suggests that Nrx-IV and Na+/K+-ATPase act together with Cora in one pathway, whereas Yrt acts in a second redundant pathway. Moreover, we found that interactions of Yrt/Cora group proteins and Crb regulate epithelial tube morphogenesis of the Drosophila respiratory system, the trachea. As previously reported for Na+/K+-ATPase, Nrx-IV and Cora, loss of Yrt causes over-elongation of multicellular tracheal tubes. Reduction of Crb dosage rescues tube size defects in all of these mutants. In addition, Crb overexpression is sufficient to increase epithelial tube length. Together, our data indicate that the antagonistic relationship between the Yrt/Cora group and Crb has a profound impact on apical-basal polarity and tracheal morphogenesis.

2840/B556

To Thine Own Cell Be True; Linking Cell Polarity to Competence during Asymmetric Fate Specification.

B. Davidson, S. Sweeney; MCDB, University of Arizona, Tucson, AZ

Cell polarization and resulting asymmetric cell divisions play an essential role in the diversification of embryonic cell fates. Here we explore how a link between cell polarity and competence may reinforce asymmetric fate specification. In embryos of the invertebrate chordate, *Ciona intestinalis*, Fibroblast Growth Factor (FGF) signaling directs an asymmetric division within four heart "founder" cells. This division results in four small heart precursor cells and four large tail muscle precursors. FGF signaling also mediates the subsequent differential specification of the heart precursor lineage. When *Ciona* embryos are dissociated and isolated heart founder cells are uniformly exposed to FGF, heart specification occurs in both daughters. This finding supports the hypothesis that differential exposure to FGF mediates differential fate specification. However, when founder cells are polarized through their initial contact with endogenous FGF, subsequent exposure to uniform FGF does not alter differential cell fate. This leads to a model in which FGF generated cell polarity affects how daughter cells respond to further FGF signaling. We are currently studying the mechanisms that generate founder cell polarity and whether this polarity directs the distribution of FGF signaling components.

2841/B557

Endocytosis Is Required for Apical Constriction during *Xenopus* Gastrulation.

J. Lee, R. Harland; Dept of Molecular and Cell Biology, University of California Berkeley, Berkeley, CA

Cell shape changes are important during normal embryonic development and abnormal disease states such as cancer. At the beginning of *Xenopus* gastrulation, bottle cells simultaneously undergo apical constriction and apicobasal elongation, transforming from cuboidal cells into elongated cells with tapered necks. How are bottle cells able to undergo dramatic shape changes quickly? We hypothesized that endocytosis of the apical membrane could regulate the efficiency of apical constriction. To investigate this, we used a biotin-labeling technique to follow endocytic activity in *Xenopus* embryos. Biotin-labeled endocytic vesicles were observed exclusively in bottle cells, suggesting that bottle cells are the only cells actively undergoing extensive endocytosis during the onset of gastrulation. Moreover, we disrupted endocytosis by expressing a dominant negative (DN) form of Dynamin, a small GTPase required for the scission of the endosome from the membrane. DN Dynamin-injected embryos exhibited shallower blastopore grooves and bottle cells that were less apically constricted, without affecting cell fate or subsequent morphogenetic events. To independently confirm that endocytosis is required for apical constriction, we injected DN and constitutively active (CA) forms of Rab5, a small GTPase required for transport of early endosomes. Injection of DN Rab5 recapitulated the DN Dynamin results, whereas CA Rab5 had no effect on either blastopore depth or apical constriction. Transcytosis does not contribute to apicobasal elongation, since cell length was not affected by the perturbation of endocytosis with DN Dynamin or DN Rab5. In summary, our results show that endocytosis is crucial for apical constriction in bottle cell morphogenesis. More generally, our findings increase our understanding of how membranes are rapidly reorganized during cell shape changes in development and disease.

2842/B558

Rotation of Cells in *Xenopus Laevis* Somites Is Dependent on Somite Boundary Formation and Coordinated Changes in Cell Polarity.

A. Price, C. Kintner; Salk Institute, La Jolla, CA

Early in development, transcription factor signaling provides cues that lead to the realization of the embryonic body plan, but how these instructions are interpreted by cells to orchestrate changes in cell shape and migration that result in morphology is less well understood. The somites are some of the earliest mesodermal structures to appear in embryos and mark the segmentation of the vertebrate axis. Patterning of the somite involves Fgf, RA, Wnt, and Notch signaling pathways and culminates in the formation of anterior and posterior somite compartments defined by gene expression and the fact that a somite boundary will only form between a posterior compartment and the subsequent anterior compartment. Disruption of compartment patterning leads to gross morphological changes such as loss of segment boundaries and disruption of somite cell fates. In *Xenopus* somites, boundary formation is followed by a coordinated change in the alignment of cells within the somite from medial-lateral (converging extending) to anterior-posterior (somite maturation). Boundary formation and cell rotation is directly preceded by Protocadherin in Neural Crest and Somites (PCNS), EphB3 and ephrinB2 expression in frog somite compartments. We have analyzed cell behavior during boundary formation and cell rotation using live explants and fluorescent cell markers and find that the behaviors in the anterior and posterior compartments of somites are distinct and depend on remodeling of the actin cytoskeleton. We show that these behaviors are necessary for the coordinated rotation of the entire somite and that changes in protocadherin or Eph/ephrin signaling disrupt boundary formation as well as these synchronized movements. Further investigations into the downstream effect of signaling on the actin cytoskeleton are ongoing.

2843/B559

Chondrocyte Polarity Defines Vectors of Cartilage Growth.

A. T. Dudley, M. J. Ahrens, Y. Li; BMBCB, Northwestern University, Evanston, IL

Bone growth is driven by cell proliferation and subsequent hypertrophy of chondrocytes arranged in columns of discoid cells that resemble stacks of coins. However, the molecular mechanisms that direct column formation and the importance of columnar organization to bone morphogenesis are not known. Here we show that discoid proliferative chondrocytes orient the division plane to generate daughter cells that are initially aligned orthogonal to the column and then intercalate. Downregulation of Frizzled (Fzd) signaling in chick long bones produces cell autonomous changes in proliferative chondrocyte organization characterized by arbitrary division planes and altered cell stacking. By contrast, in Piga mutant mice, chondrocytes display normal planes of cell division but fail to intercalate into columns. Thus, orientation of cell division and cell intercalation are mechanistically separable events. In all cases, defects in column formation lead to shorter wider bones, consistent with a model in which cell arrangement defines vectors of growth in developing cartilage. Collectively, our findings demonstrate that signaling pathways regulate cell polarity and cell arrangement in the cartilage growth plate and suggest important roles for polarized cell behaviors in generating the unique morphological characteristics that shape individual cartilage elements of articulated skeletons.

2844/B560

Noncanonical Frizzled Dependent Signaling Controls Chondrocyte Polarity during Cartilage Growth.

Y. Li; Northwestern University, Evanston, IL

A central question in developmental biology is how morphologically distinct structures are generated from similar cell types. The skeleton provides a good model system to answer this question due to the highly diverse and complex morthological differences displayed by individual elements. Analysis of the growth plate cartilage by Dodd suggested that longitudinal growth of long bone might result from the specific arrangement of chondrocytes. In the proliferative zone, chondrocytes become discoid and arrange in columns, like stacks of coins, which are parellel to the long axis of the cartilage. Although well-organized chondrocytes derive from a relatively unstructured pool of progenitor cell, it is not known whether this arrangement of cells is the result of a regulated process or is in response to physical constraints of the cartilage matrix. Here we test Dodds' model that cell columns in the growth plate cartilage form from a single progenitor cell by a process in which cells divide orthogonal to the stack then rearrange. Our work has uncovered a central role of noncanonical Frizzled dependent signaling in the regulation of chondrocyte polarity and morphogenesis in the growth plate cartilage. Funding for these studies was provided by the searle Leadership Fund of the Chicago Community Trust.

2845/B561

Planar Cell Polarity in Ciliated Epithelial Cells.

E. Vladar, J. Axelrod; Pathology, Stanford University, Stanford, CA

Multiciliated airway epithelial cells contain hundreds of cilia that beat together in a whip-like motion to move mucus and debris away from the lungs, which is essential for airway function. Cilia must be physically oriented in the direction of the beat for proper beating, but how they do this is unknown. The Planar Cell Polarity (PCP) signaling mechanism, which orients cells and cellular structures within organs and along the body axis, is an ideal candidate for regulating ciliary polarity. To investigate the involvement of the PCP pathway, we have determined the expression profile and localization of core PCP proteins in mouse trachea and cultured tracheal epithelial cells. Our results suggest that the PCP pathway is active in these cells and functions in a conserved manner. We are using transgenic mice lacking PCP proteins and lentiviral RNAi and overexpression in cultured cells to test whether they are required for polarized ciliary orientation. These results will guide further experiments to uncover the mechanism through which this pathway orients cilia. This research will help elucidate the PCP pathway, defects in which are associated with multiple serious health problems including spina bifida, congenital deafness and polycystic kidney disease.

2846/B562

The Role of the Planar Cell Polarity (PCP) Pathway in Generating Multi-Ciliate Cell Polarity.

B. Mitchell[2], C. Kintner[1]; [1]Molecular Neurobiology, Salk Institute for Biological Studies, La Jolla, CA, [2]Cell and Molecular Biology, Northwestern University Feinberg School of Medicine, Chicago, IL

An interesting form of cellular planar polarity is the ability of ciliated epithelia to generate directed fluid flow. To achieve this, multi-ciliate cells must orient the beating of their cilia in the same direction. Using mean orientation of cilia within a cell as a marker for a cells polarity we have assessed the role of the non-canonical Wnt / Planar Cell Polarity (PCP) pathway in establishing the planar polarity of the ciliated epithelia of the Xenopus larval skin. Our data provides a developmental context for the generation of ciliated cell polarity and leads to a two step model for how this is achieved. First, the non-ciliated outer epithelium is polarized. Second, as naive ciliated cells intercalate into the outer epithelia they acquire polarity information from the previously polarized outer cells. Significantly, we have developed an assay for generating clones of mutant cells in Xenopus skin and have used this assay to demonstrate the presence of domineering non-autonomy for the trans-membrane PCP components Strabismus / Van Gogh and Frizzled-3 but not for the intracellular component Disheveled. Consistent with results from Drosophila, Strabismus and Frizzled have reciprocal domineering effects on cell polarity. The study of ciliated cell orientation provides a novel paradigm for the study of vertebrate planar polarity and allows for a detailed analysis of PCP signaling.

2847/B563

Regulation of Cdc42-Mediated Epithelial Morphogenesis in 3D.

J. Durgan[1], A. Jaffe[2], N. Kaji[1], A. Hall[1]; [1]Cell Biology, MSKCC, New York, NY, [2]Developmental and Molecular Pathways, Novartis Institutes for Biomedical Research, Cambridge, MA

Defects in morphogenesis underlie a range of developmental syndromes and disruption of epithelial tissue is a hallmark of late stage cancer progression. Recent work in our laboratory has exploited a model for studying epithelial morphogenesis in 3D tissue culture, using Caco-2, a human intestinal cell line. Single cells, seeded on top of, or embedded within, a collagen/matrigel mix proliferate and self-organize to form 3D cysts over a period of days. Each structure comprises a single layer of cells, in which the basal surfaces are in contact with the extra-cellular matrix, while the apical surfaces surround a fluid filled lumen. The architecture of the Caco-2 cyst bears many hallmarks of a physiological, intestinal epithelium and thus it represents a powerful model for analyzing the morphogenetic program. Using RNAi-based approaches, we have established that Cdc42 plays a critical role during Caco-2 morphogenesis in 3D. Cdc42 depletion manifests in the disruption of mitotic spindle orientation and the development of an aberrant structure containing multiple lumen. Interestingly, each lumen is surrounded by apically polarized aPKC, suggesting that Cdc42 is not required to establish apical-basal polarity per se, but rather to correctly position the apical surface. To determine the mechanism through which Cdc42 is regulated during Caco-2 morphogenesis, we have performed RNAi-based screens against the human GEFs and GAPs. Work is ongoing to characterise the signalling events both upstream and downstream of Cdc42 which control epithelial morphogenesis.

2848/B564

JNK-Dependent Regulation of Polarization in 3-D Mammary Epithelial Cell Assemblies.

J. Whyte, L. Thornton, F. Martin; UCD Conway Institute, University College Dublin, Dublin, Ireland

Mouse mammary epithelial cells organize as spherical acini when grown on laminin-rich ECM. Acinus formation begins with the apicobasal polarization of the outer cells of the assembly and the withdrawal of these cells from the cell cycle. Subsequently, the internal cells die by apoptosis and form a hollow lumen. Polarization in an epithelial sheet is initially established by the generation of an active signaling complex consisting of atypical PKCzeta, Par3 and Par6. We have previously shown that JNK activity is necessary for functional acinus formation and we now examine JNK-dependent regulation of polarization. We demonstrate that PKCzeta is phosphorylated (at T410) and activated in the early stages of acinus formation in a JNK-dependent fashion. Upstream of PKCzeta, cdc42 is activated early in acinus formation and forms a complex with Par3. This complex is not observed when JNK activity is inhibited. Inhibition of PKCzeta activity using either drug treatment or lentiviral delivery of dominant negative PKCzeta constructs; is sufficient to disrupt the assembly of organized acini. We observe an increase in expression of the basolateral polarity complex proteins; scrib, dlg and lgl during the early stages of acinus formation. Their expression is significantly reduced in JNK-inhibited spheres, but not through the repression of c-jun-mediated transcription. We can immunoprecipitate scrib from JNK-inhibited spheres with antibodies against ubiquitin, suggesting that these proteins undergo ubiquitin-mediated degradation. We therefore propose that JNK regulates key molecular events leading to the activation of PKCzeta and the expression of key polarity proteins, which are necessary for early events in cell polarization.

2849/B565

Deregulation of the Polarity Gene Scribble Disrupts Morphogenesis Inhibits Cell Death and Promotes Myc-Induced Mammary Tumorigenesis.

L. Zhan[1], A. Rosenberg[1], C. Allred[2], S. Muthuswamy[1]; [1]Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, [2]Department of Pathology and Immunology, Washington University School of Medicine, St Louis, MO

Loss of cell polarity proteins such as Scribble induces neoplasia in Drosophila by promoting uncontrolled proliferation. The role polarity pathways play during tumorigenesis in mammals is poorly understood. We demonstrate that deregulation of Scribble in mammary epithelia disrupts polarity, blocks normal three-dimensional morphogenesis, inhibits apoptosis and induces development of dysplastic mammary outgrowths in vivo that progress to tumors after long latency. Downregulation of expression of Scribble strongly cooperates with oncogenes such as Myc to transform 3D acini and induces tumors in vivo by blocking Myc-induced activation of an apoptosis pathway. Mislocalization of Scribble from cell-cell junctions phenocopies loss of expression demonstrating that proper localization is required for Scribble to promote morphogenesis and block transformation. We also show that Scribble is downregulated and/or mislocalized in mouse mammary tumors and in human breast cancers. Thus, deregulation of Scribble is selected-for during spontaneous tumorigenesis and promotes transformation of epithelia in mammals by disrupting polarity, blocking morphogenesis and inhibiting apoptosis.

2850/B566

Disruption of Apical Polarity in Mammary Epithelium Is Associated with the Transcriptional Down-Regulation of Genes Linked to Cellular Homeostasis and Breast Cancer.

C. Plachot[1], J. A. Stanley[1], L. An[2], H. Adissu[1], R. W. Doerge[2], S. Lelievre[1]; [1]Basic Medical Sciences, Purdue University, West Lafayette, IN, [2]Statistics, Purdue University, West Lafayette, IN

Differentiation of monolayered epithelia is characterized by the formation of a basoapical polarity axis. In ducts and glands, apical polarity results from the positioning of tight junctions at the apex of cells, against the lumen. Apical polarity is altered early on during cancer development. Using mammary glandular structures (acini) produced in three-dimensional cell culture we have demonstrated that, in order for mammary epithelial cells to exit quiescence and enter the cell cycle, acini have to lose apical polarity. Our hypothesis is that a set of genes dependent on apical polarity status is controlling quiescence and possibly other important features involved in tumorigenesis. Using Affymetrix technology we have performed microarray analysis on the 22,277 features/genes of the Human Genome U133A 2.0 array Chip with four biological replicates of mammary acini with full basoapical polarity compared to acini treated with two different methods to specifically alter apical polarity. “AffyRNAdeg” and “plotAffyRNAdeg” bioconductor plots showed no significant RNA degradation for all the samples used. After confirming that probe intensities were comparable among chips within the same sample type (four replicates for each condition) using box plot, PM density plots, and pair-wise MA plots, a robust local regression was applied to normalize the background corrected data (i.e., to reduce the non-linear relationship among chips). An analysis of variance (ANOVA) was employed to detect statistically significant differentially expressed genes between two samples using the normalized data. The type I error rate was adjusted using a Holm’s sequential Bonferroni correction procedure, with a significance level of 0.05. Results from this statistical analysis resulted in 30 genes that were found to be commonly and significantly down-regulated in cells with altered apical polarity compared to control. Among these genes several have been shown to be transcriptionally down-regulated in breast cancer and/or are related to proliferation control.

2851/B567

Characterization of a MARVEL-dependent Trafficking Machinery in MDCK Cyst Lumen Formation.

A. E. Rodriguez-Fraticelli, J. F. Aranda, M. A. Alonso, F. Martin-Belmonte; Centro de Biologia Molecular Severo Ochoa, Madrid, Spain

Madin-Darby Canine Kidney (MDCK) cells grown on extracellular matrix gels develop onto three dimensional cysts in which cells acquire the polarized epithelial phenotype and organize in a monolayer surrounding a fluid filled cavity. The 3D-MDCK system is an excellent model for studying cell polarity signaling pathways due to its resemblance to physiological in vivo conditions. Cyst lumen formation requires apical vesicle transport and membrane fusion events, but the trafficking machinery responsible for these processes remains unknown. In other cellular models, membrane fusion and apical trafficking are regulated by MARVEL domain containing proteins. We have studied the cellular distribution and function of a MARVEL family protein, MAL2, in cyst morphogenesis. The canine MAL2 cDNA was cloned using specific RT-PCR and then sequenced. A specific antibody was generated against an N-terminal peptide and specificity was assayed both in Western blot and Immunofluorescence experiments. The subcellular localization of MAL2 was then determined by using either antibodies or fluorescent protein chimeric constructs in stable expressing cell lines. In fully developed cysts, MAL2 localizes in the apical pole of cells and showed marked colocalization with recycling endosome marker GTPase, Rab11. Throughout the morphogenetic process, MAL2 resides in an intracellular compartment which reallocates together with the aapical secretion machinery when cells become polarized. During transcytosis

of apical marker Gp135, which is needed for lumen formation, we observe extensive colocalization with MAL2. At the light of these results we propose that MARVEL domain containing proteins, such as MAL2 may be relevant for cyst morphogenesis.

2852/B568

Mechanisms of Lumen Formation in Multicellular Epithelial Cysts: Modulation by Small GTPases.

D. Bryant, A. Datta, K. Mostov; University of California San Francisco, San Francisco, CA

The ability to generate distinct apical and basolateral cell surfaces is a fundamental requirement for epithelial cells to organize into multicellular organs. In the kidney, the apical surface provides the lumen of ductal epithelium, whilst the basolateral surface faces the internal milieu. Using 3-dimensional MDCK cell culture conditions, we are analyzing the trafficking and signaling pathways that modulate apical polarity, membrane transport and lumen formation. We previously demonstrated that the small GTPase Cdc42 plays a central role in linking apical membrane formation, the conserved Par6/aPKC polarity complex, and spatial segregation of phosphoinositide species to generate epithelial lumens (Martin-Belmonte et al. Cell. 2007). Here, we characterize a membrane transport route for generation of apical lumen formation, dependent on Rab11a and Rab8a GTPase complexes. Both Rab8a and Rab11a are required for apical transport, and polarity orientation to form a single lumen. We also demonstrate crosstalk between Rab-mediated vesicle transport, and the Cdc42/Par6/aPKC polarity complex. Perturbation to GEF- or GAP-mediated Rab activation, Rab effector proteins, or the polarity complex inhibits the ability of cysts to form single lumens. These findings are important for understanding how luminal structures are generated in epithelial organs, and provide insight into how membrane traffic may be deregulated in glandular neoplasia and renal pathologies.

2853/B569

The Molecular Basis for Interkinetic Nuclear Oscillations in the Mammalian Neuroepithelium.

J. Tsai[1,2], W. Lian[1], R. Vallee[1]; [1]Pathology and Cell Biology, Columbia University College of Physicians and Surgeons, New York, NY, [2]Neurobiology Program, Skirball Institute of Biomolecular Medicine, New York University Medical Center, New York, NY

The vertebrate CNS originates from a neuroepithelium composed of highly asymmetric neuroepithelial and, subsequently, radial glial progenitor cells. These cells give rise to virtually all of the neurons and glia of the brain, and neural stem cells in the adult. Their nuclei undergo a remarkable series of "interkinetic" oscillations, with division occurring only at the ventricular, apical, cell surface. How the nuclei move, and how the direction and timing is regulated has remained a mystery for the past half century. Using *in utero* electroporation of shRNA-encoding cDNAs we previously found that knockdown of the lissencephaly gene, LIS1, completely blocks the interkinetic nuclear oscillations, as well as the subsequent radial migration of committed neuronal precursors (*J Cell Biol*, **176**:935). Live imaging of coexpressed histone, centrosome, and microtubule plus-end markers revealed that LIS1 and dynein are required for both nuclear and centrosome movement in the radially migrating cells (*Nat Neurosci*, **10**:970). We have now applied these approaches to the behavior of the neuroepithelial and radial glial cells. We find apically-directed nuclear movement (toward the ventricle) is strictly unidirectional, rapid (1-2 μm/min), and very discontinuous. Basally-directed movement is also strictly unidirectional, but very slow (~0.2 μm/min) and continuous. Centrosomes imaged live using DsRed-centrin remained at the ventricular surface throughout the nuclear oscillatory cycle, but split and separated during mitosis. Microtubule plus ends imaged live using GFP-EB3 and GFP-tubulin revealed growing microtubules throughout the entire 1-2 mm length of the cell uniformly oriented toward its basal end. During mitosis, however, microtubules were completely depleted from the basal process, which, however, persisted as a thin attachment to the pial membrane. Inhibition of myosin II with blebbistatin by RNAi had no effect on the nuclear oscillations. Based on these observations, we propose that apically- and basally-directed interkinetic nuclear movements in this and other pseudostratified epithelia involve distinct mechanisms using opposite-directed microtubule motors specifically activated during G1 or G2. Supported by HD40182.

2854/B570

A Positive Feedback Loop between Ras and PI3K Regulates Neuronal Symmetry Breaking and Axon Formation.

M. L. Fivaz[1,2], S. Bandara[2], T. Inoue[3,2], T. Meyer[2]; [1]Neuroscience and Neurobehavioral Disorders Program, DUKE-NUS Graduate Medical School Singapore, Singapore, [2]Chemical and Systems Biology, Stanford University, Stanford, CA, [3]Cell Biology, Johns Hopkins University, Baltimore, MD

Neuronal polarity is initiated by a symmetry-breaking event whereby one out of multiple minor neurites undergoes rapid outgrowth and becomes the axon. Axon formation is regulated by phosphatidylinositol 3-kinase (PI3K)-related signaling elements that drive local actin and microtubule reorganization, but the upstream signaling circuit that causes symmetry breaking and guarantees the formation of a single axon is not known. Here, we use live FRET imaging in hippocampal neurons and show that the activity of the small GTPase HRas, an upstream regulator of PI3K, markedly increases in the nascent axonal growth cone upon symmetry breaking. This local increase in HRas activity results from a positive feedback loop between HRas and PI3K, locally reinforced by vesicular transport of HRas to the axonal growth cone. Recruitment of HRas to the axonal growth cone is paralleled by a decrease in HRas concentration in the remaining neurites, suggesting that competition for a limited pool of HRas guarantees that only one axon forms. Mathematical modeling demonstrates that local positive feedback between HRas and PI3K, coupled to recruitment of a limited pool of HRas, generates robust symmetry breaking and formation of a single axon in the absence of extrinsic spatial cues.

2855/B571

Par6 Alpha Controlled Myosin II Motors Orchestrate the Coordinated Translocation of Centrosome and Soma During Glial Guided Neuronal Migration.

D. Solecki; St. Jude Children's Research Hospital, Memphis, TN

The emergence of the laminar architecture in cortical regions of the vertebrate brain depends on the directed neuronal migration along glial fibers. We previously showed that the conserved polarity protein Par6α localizes to the centrosome and controls cytoskeletal dynamics required for the coordinated forward movement of the centrosome and neuronal soma. The cytoskeletal components that produce this unique form of cell polarity and their relationship to Par6α are unknown. Here we use time-lapse imaging in primary cerebellar granule neurons to

show that non-muscle Myosin II, an actin-based cytoskeletal motor, localizes in the base of the leading process just forward of centrosome in migrating neurons. As the neuron moves, a region of high Myosin II motor activity is observed near the front of the cell soma. Activation of acto-myosin contractility increased the speed of centrosomal and somal translocation, while inhibition retarded the coordinated movement of centrosome and soma. Thus, Myosin II motors provide the force necessary to execute the program of polarity in migrating CNS neurons. Ectopic expression of Par6α inhibited regulatory myosin light chain phosphorylation by a mechanism that involved lowered ROCK1 phosphorylation of Myosin Light Chain Phosphatase. These findings suggest that conserved polarity proteins control local acto-myosin contractility and provide new mechanistic insight into the cellular and molecular targets of the PAR complex in the control of neuronal polarity and morphogenesis.

2856/B572

Multiple Domains in the Crumbs Homolog 2a (Crb2a) Protein Are Required for Its Localization and Function in Rod Photoreceptors.

A. Jensen[1,2], Y. Hsu[1,2]; [1]Biology, University of Massachusetts, Amherst, MA, [2]Molecular and Cell Biology Graduate Program, University of Massachusetts Amherst, Amherst, MA

Vertebrate retinal photoreceptors are morphologically complex cells that have two apical regions, the inner segment and the outer segment. The outer segment is continuously renewed throughout the life of the organism. The molecular and cellular mechanisms that underlie vertebrate photoreceptor morphogenesis and the maintenance of the outer segment remain unknown. The Crumbs (Crb) complex is a key regulator of apical membrane identity and size in epithelia and Drosophila photoreceptors. Mutations in the human gene *CRUMBS HOMOLOG 1* (*CRB1*) are associated with early and severe vision loss. In order to begin to understand the role of the Crb family of proteins in vertebrate photoreceptors we generated transgenic zebrafish in which rod photoreceptors overexpress full-length Crb2a protein and several Crb2a proteins that lack specific domains. We examined the localization of these Crb2a constructs and their effects on rod morphology using confocal microscopy. Only full-length Crb2a protein that retains all domains approximated the normal localization of Crb2a protein and localized to the inner segment. Several constructs that lack the extracellular domain localize to the outer segment. Overexpression of several constructs led to a modest increase in outer segment size. Interestingly, overexpression of full-length Crb2a greatly increased inner segment size, and the increased size appears due to a massive increase in the number and density of ribosomes.

Stem Cell Biology (2858 – 2881)

2858/B574

Astrocyte Precursor Cells in the Developing Mouse Cerebellum.

N. Cai, M. Kurachi, Y. Ishizaki; Dept. Mol. Cell. Neurobiology, Gunma University Graduate School of Medicine, Maebashi, Japan

It is generally considered that neural stem cells are committed to become either neuronal precursor cells or glial precursor cells before terminally differentiating into neurons or glial cells. Up to now, neuronal precursor cells and oligodendrocyte precursor cells have been identified in several areas in the murine CNS. Presence of astrocyte precursor cells (APCs), however, has been reported only in the developing rat optic nerve so far (Mi & Barres, 1999). In this study, we report isolation of APCs from the developing mouse cerebellum. Cerebella from early postnatal mice were digested with papain, and the dissociated cells were fractionated by a step gradient of 35% and 60% Percoll. The small cell fraction was mainly comprised of granule cell precursors, while the large cell fraction contained the GFAP-positive cells and the GFAP-weakly-positive cells. The large cell fraction was subjected to immunopanning with the A2B5 antibodies to select the GFAP-weakly-positive cells, as most of the GFAP-weakly-positive cells were A2B5-positive, while most of the GFAP-positive cells were A2B5-negative. In the presence of both basic fibroblast growth factor (bFGF) and bone morphogenetic protein 4 (BMP4), many of the cells thus obtained incorporated BrdU, and most of the BrdU-positive cells were GFAP-weakly-positive, suggesting that the combination of bFGF and BMP4 is essential for the proliferation of these cells. When BMP4 or bFGF was withdrawn from the culture, many of these cells underwent apoptosis, suggesting these factors are also essential for their survival. When these cells were treated with ciliary neurotrophic factor (CNTF) or leukemia inhibitory factor (LIF), the proportion of GFAP-positive cells increased dramatically, suggesting these cells are APCs and their astrocytic differentiation is induced by these factors. There are three types of astrocytes in the murine cerebellar cortex, Bergmann glia, protoplasmic astrocytes in the granule layer, and fibrous astrocytes in the white matter. Characterization of APCs may prove useful for the elucidation of the mechanism for the generation of these astrocytes during cerebellar development.

2859/B575

Role of FGF Signaling and Extracellular Microenvironment in Stem Cell Development of the Mammary Gland.

P. Lu, A. Sharma, Q. Xue, Y. Magan, Z. Werb; Anatomy, UCSF, San Francisco, CA

A major focus in mammary gland biology has been to understand the mechanisms, especially regarding the role of the extracellular matrix (ECM) microenvironment, governing development of mammary stem cells (MSCs), which can self-renew, differentiate and give rise to the whole gland. Fibroblast growth factor (FGF) signaling has long been associated with the mammary gland. Indeed, several founding members of the FGF family were first discovered because their loci are frequently targeted by mouse mammary tumor virus (MMTV), whose insertion causes excess FGF signaling activity and breast tumors. Moreover, FGF signaling is required for embryonic development and for postnatal branching morphogenesis in the mammary gland. To test the hypothesis that FGF signaling is required for normal development of MSCs and excess FGF signaling contributes to formation of cancer stem cells (CSCs) and breast cancer, we have used a combination of in vitro and in vivo analyses to examine the consequences of loss or gain of FGF signaling activities on MSC development. We established a protocol to enrich for MSCs based on expression of aldehyde dehydrogenase from mouse mammary gland. We found that, unlike other cells, MSCs readily underwent acinar morphogenesis in 3D cultures. Interestingly, loss of FGFR2 function compromised the ability of MSCs to form acini in vitro. Moreover, unlike their normal counterparts, MSCs lacking FGFR2 were unable to regenerate the mammary gland when they were transplanted into the mammary fat-pad. Together, these data demonstrate that FGF signaling is required for normal development of

MSCs. We are currently investigating how the ECM modulates FGF signaling as a stem cell niche. In addition, we are analyzing how the Wnt signaling, which is also critical for MSCs and often targeted by MMTV insertions, synergizes with FGF signaling to regulate normal MSC development, whether they do so by modulating the ECM, and how their deregulation promotes formation of cancer stem cells and breast cancer.

2860/B576

Subcellular Architecture of a Germline Stem Cell Niche.

D. T. Byrd[1,2], K. Knobel[1], K. Schmitt[1], A. Amasino[1], J. Kimble[1,2]; [1]Biochemistry, University of Wisconsin-Madison, Madison, WI, [2]Howard Hughes Medical Institute, Madison, WI

A stem cell's decision between self-renewal and differentiation is governed by extrinsic stimuli from the surrounding microenvironment, or niche, as well as by intrinsic cues. In *C. elegans*, the distal tip cell (DTC) provides a niche for germline stem cells and maintains a "mitotic region" by Notch signaling. DTC number and dosage of the LAG-2 DSL ligand, which is expressed by the DTC, can affect the number of germ cells in the mitotic region. To investigate DTC niche function, we made a panel of fluorescent protein markers to visualize its subcellular structure *In Vivo*. These include markers for the nuclear envelope, endoplasmic reticulum, Golgi, endosomes, and plasma membrane. Using membrane-associated markers, we found that contact between the DTC niche and germ cells in the mitotic region is extensive. The extent of the processes defines the "niche region", which includes ~60 germ cells. Formation and maintenance of the DTC processes is dependent on mitotically-dividing germ cells, suggesting germ cells signal to the niche-forming DTC. The intimate contact between the niche and germ cells may provide a mechanism to physically anchor the distal-most germ cells within the niche and/or provide localized signaling to maintain an undifferentiated and immature stem cell-like state among germ cells in its grasp.

2861/B577

Screening for New Factors for Reprogramming.

B. Feng[1], J. Jiang[1, 2], P. Kraus[3], Y. Loh[1, 2], W. Zhang[1, 2], J. Ng[1], J. Heng[1], V. Cacheux-Ratabou[1, 4], B. Lim[3], T. Lufkin[3], H. Ng[1, 2]; [1]Gene Regulatory Laboratory, Genome Institute of Singapore, Singapore, [2]Dept of Biological Sciences, National University of Singapore, Singapore, [3]Stem Cell and Developmental Biology, Genome Institute of Singapore, Singapore, [4]Cancer Biology & Pharmacology, Genome Institute of Singapore, Singapore

The developmentally restricted fibroblasts can be reversed to a pluripotent state (e.g. reprogramming) using retrovirus-mediated transduction of four transcription factors such as Oct4, Sox2, Klf4 and c-Myc. Here, we screened a panel of new candidate factors in the presence of any three of the original 4 factors, aiming to find alternative reprogramming factors and further dissect mechanism of this process. Totally, we tested 18 factors (transcription factors and chromatin modifiers) using direct reprogramming of genetically unmodified fibroblasts as described previously. Surprisingly, all 9 tested chromatin modifiers showed no effect in either facilitating the reprogramming or in replacing any of the original 4 factors. On the other hand, the Klf2 and Klf5 exhibit certain activity in replacing the closely related Klf4 for reprogramming, whereas no effect was detected with Klf10 among the same family.

2862/B578

Characterization of Human Mesenchymal Stem Cell Neuronal Differentiation by SILAC and High Content Screening Analysis.

D. Hughes[1], S. Hong[1], M. Rosenblatt[1], B. Krastins[2], D. Sarricino[2], M. Lopez[2], A. Kamath[3], R. Ghosh[1], J. Rogers[1]; [1]Thermo Fisher Scientific, Rockford, IL, [2]Thermo Fisher Scientific, Cambridge, MA, [3]Cellular Engineering Technologies, Coralville, IA

Multipotent stem cells hold great promise for regenerative medicine and as important tools for biological research. Adipose-Derived Human Mesenchymal Stem Cells possess the ability to differentiate into multiple cell lineages and are readily available in sufficient quantity, making them an attractive tool to study differentiation. However, a full understanding of the regulation of differentiation at the protein level remains unknown. The goal of this study was to use proteomics and cell-based screening tools to characterize stem cells during neuronal differentiation. To discover differentially regulated proteins, we used Stable Isotope Labeling of Amino acids in Cell culture (SILAC) with subsequent proteomic analysis by mass spectrometry. Simultaneously, we used high content screening (HCS) to assess neurite outgrowth and temporal-spatial expression changes of known neuronal markers. Here we report the significant regulation of many gene ontologies identified by SILAC quantitative proteomics. These changes were accompanied by an increase in both neuronal morphology and levels of protein markers MAP2 and Beta-3-tubulin as determined by HCS analysis. Both models demonstrate how proteomic and cell-based profiling of stem cells offer valuable insights into the mechanisms of cellular differentiation.

2863/B579

Characterization of Cultured C-Kit Positive Cardiac Stem Cells Derived from Adult Rat Hearts.

N. Kawaguchi[1], S. Miyamoto[1,2], G. Ellison[3], K. Hasan[1], R. Matsuoka[1]; [1]IREIIMS, Tokyo Women's Medical University, Tokyo, Japan, [2]Cardiovascular Surgery, Tokyo Women's Medical University, Tokyo, Japan, [3]Stem Cell and Molecular Physiology, Research Institute for Sport and Exercise Science, John Moores University, Liverpool, United Kingdom

[Objective] Previously it has been shown that the adult rat heart contains stem cells that can differentiate into the three cardiac lineages, both in vitro and in vivo (Beltrami et al., Cell, 2003). We consider that the usage of cardiac stem cells (CSC) for heart failure therapy would be beneficial. In this study we were interested to investigate the issue of safety of CSC-based therapy by determining whether CSCs isolated from the adult rat heart, exhibit the same characteristics between isolations and whether they retain their characteristics over time in culture. [Methods] We isolated c-kit positive CSCs from adult rat hearts using AUTOMACS more than 10 times and cultured each CSC-Bulk Culture (CSC-BC) population until 40 passages. We monitored CSC-BCs for the expression of stemness and cardiac differentiation markers as well as their potential for differentiation into other lineages, such as adipocyte and mesenchymal. The various marker genes and proteins were detected using RT-PCR and immunocytochemistry. [Results] We found that CSC-BCs cultures were mixtures of primitive, undifferentiated stem cells and committed progenitor cells, not only for the cardiac, but also for adipocyte and skeletal muscle lineages. The expression level of GATA-4, one of the cardiac myocyte markers, was variable between CSC-BCs and we found that some CSC-BCs could form spheres

(cardiospheres). Furthermore, when CSCs were co-cultured with adult rat cardiac myocytes, we found increased differentiation into cardiac myocyte-like cells as well as increased survival of the adult rat cardiac myocytes. The growth factor, IGF-1 was up-regulated in the culture medium. [Discussion] Though our results suggest that CSCs are multi-potent by nature, their phenotypic and genotypic properties are variable between cultures and unstable over long-term culture. Our results also indicate that CSCs have the potential to enhance the survival of cardiac myocytes perhaps by secreting paracrine factors. [Conclusion] CSC-BCs could be an efficient source of cardiac progenitors. However, they can also spontaneously differentiate into other cell types in vitro, suggesting long time culture may cause unwanted differentiation.

2864/B580

Proteomic Analysis of Single Colony-Derived Strains from Human Mesenchymal Cells of Dental Pulp Origin.

V. V. Borutinskaite[1], G. Treigyte[1], J. Savickiene[1], K. Magnusson[3], A. Pivoriunas[2], R. Navakauskiene[1]; [1]Department of Developmental Biology, Institute of Biochemistry, Vilnius, Lithuania, [2]Department of Experimental Research, Institute of Experimental and Clinical Medicine, Vilnius, Lithuania, [3]Department of Experimental and Clinical Medicine, Linkoping University, Linkoping, Sweden

Deciduous teeth have robust stem cells in their dental pulp. Such teeth may serve as an easily obtainable alternative to embryonic stem cells, the use of which has proved controversial. These stem cells are multipotent and capable of differentiating into different kinds of cells, such as osteoblasts, chondrocytes, adipocytes and neural-like cells. Our aim was to isolate pluripotent stem cells from human dental pulp and examine the protein patterns of clonal cell lines, each originating from a single cell of deciduous pulp. We found that all cells had typical fibroblastoid morphology and good proliferating activity. One clone (F5) demonstrated a potential to generate neural-like tissue. Using protein separation by 2D technology and mass spectrometry analysis we identified proteins that were conservatively expressed in all clones, and also proteins that expression level change between different clones. The identified proteins could be divided into several groups based on their function within the cell. First, a group of proteins such as pyruvate kinase 3 and TPI1 important energy providing enzymes that are important for the cell growth, metabolism and survival. Second, a group including beta- and gamma-actins, beta-tubulin , vimentin, alpha-actinin4 and septin2, proteins that are important for cell cytoskeleton formation. Third, a group of chaperons (BiP, Hsc70, CCT3), that are involved in protein folding. We observed that the F5 clone expressed fibronectin 1 (FN), which is known to be up-regulated during neuronal differentiation. Moreover, we detected a down-regulation of vimentin, a mesenchymal cells marker in this clone. It is known, that developing neurons replace their vimentin-rich cytoskeleton with one with more neurofilaments. In conclusion, our data demonstrate differences in the protein patterns of clones derived from dental pulp. It likely reflects heterogeneity among the stem cells isolated from dental pulp, making it very attractive as alternative source of pluripotent stem cells within human organism.

2865/B581

Expression Profiling Analysis of hMSCs in Early Events of Chondrogenesis by Comparative Proteomics and Biological Function Explore of Type VI Collagen in Early Chondrogenesis In Vitro.

J. Cui[2, 1], Y. Liu[2], Y. Ohyabu[1], T. Uemura[1]; [1]Nanotechnology Research Institute, AIST (Natl Inst. Adv. Industr. Sci. Technol.), "Tsukuba, Ibaraki", Japan, [2]Institute of Biomedical Sciences, Fudan University, Shaghai, China

To better understand the initiation mechanism of chondrogenic differentiation and discover more potential key proteins or factors associated with lineage decision and early events of chondrogenesis, a novel in vitro RWV differentiation culture system has successfully been developed to direct hMSCs into chondrogenic differentiation in this study, and then the entire proteome of undifferentiated hMSCs and spherical constructs during early events of chondrogenesis were comparatively analyzed with 2-DE and MALDI-TOF-MS/MS. Thirty-one differential candidate proteins with high confidence were finally identified. In comparison with un-differentiated hMSCs, six of the identified proteins were significantly up-regulated in spherical constructs on day10, while the other 25 were down-regulated in spherical constructs on day10. Two differential matrix proteins COL6A1 and COL6A2, which were found to be up-regulated distinctly in the early events of chondrogenesis in parallel with the increase of SOX9, drew our interest for further investigation. A COL6-specific antibody supplemented in induction media effectively postponed the progress of early chondrogenesis, followed by the successive reduction of extracellular COL6 though a temporary increase in genes expression and upregualtion of SOX9 phosphorylation at residue 181 at the early stage of neutralization. All results suggested that the extracellular matrix protein COL6 might positively regulate the expression level of SOX9 in the early events of chondrogenesis through ECM-integrins mediated signal transduction, and further modulate the rate and progress of early chondrogenesis.

2866/B582

Gene Expression Profile in the Adipogenesis Induction of Dental Pulp Cells.

T. Nozaki, K. Ohura; Parmacology, Osaka Dental University, Osaka, Japan

Objective: We previously reported that rat dental pulp-derived cells were able to differentiate into an osteoblast lineage. To further evaluate the multipotency of the cells and elucidate the genes regulation in the differentiation, we examined their *in vitro* differentiation into an adipocyte lineage. Methods: Dental pulp cells derived from five-week-old male Wistar rats were gently separated from the posterior of the mandible using dental reamers. *In vitro* differentiation induction assays were performed by applying *in vitro* conditions previously used to induce human bone marrow cell differentiation. Specifically, insulin, 3-isobutyl-1-methylxanthine and dexamethasone were added as adipogenesis-inducing agents. The degree of differentiation was evaluated by quantitative analysis of Oil Red O staining at 0 (pre-induction), 1, 2 and 3 weeks after induction. Using GeneChip Rat Expression Array (Affymetrix Inc., USA) containing 31042 probes, gene expressions were comprehensively compared and analyzed. Quantitative analysis of gene expression was performed using real-time RT-PCR to elucidate gene regulation in the potential adipogenic lineage. Results: At 3-weeks after the induction, the cells containing lipid droplets in their cytoplasm were observed, and the measured absorbance of the Oil-Red-O staining was significantly increased ($p<0.05$). Based on the analysis of gene expression profile, 3418 probes were extracted after filtering. Ten clusters were gotten from the heatmap containing the genes that showed a difference in 1, 2, and 3W, compared with 0W. The frequency which appeared in KEGG pathway was analyzed to know for what kind of pathway, which is included significantly and abountly in differentiation. Notably, the genes on Ppar gamma pathway, Ppar

gamma, C/EBP family and Fabp4 (aP2) were up-regulated by more than 3-fold. Conclusions: The rat dental pulp-derived cells showed the potential to differentiate into an adipocyte lineage following *in vitro* induction. The up-regulation of Ppar gamma pathways is proposed as a key signal transduction pathway in the differentiation. This study was supported, in part, by JSPS. KAKENHI (18592211) and by MEXT. HAITEKU (2007-2011).

2867/B583

Characterization of the Early Embryo upon Loss of Histone Methyltransferase Setd1a.

A. S. Bledau[1], A. F. Stewart[2], K. Anastassiadis[1]; [1]Centre for Regenerative Therapies Dresden, Dresden, Germany, [2]Genomics, Dresden University of Technology/BioZ, Dresden, Germany

Epigenetic mechanisms are essential in establishing and maintaining gene expression patterns during embryonic development. Posttranslational modifications of specific histone residues within chromatin have been implicated in both activation and silencing of gene expression. Trithorax group (trxG) proteins specifically methylate histone H3 at lysine 4 (H3K4), a histone modification that correlates with active gene expression. However, the mechanisms of H3K4 methylation in gene activation and cell fate determination are still unclear. There are six functional H3K4 methyltransferases expressed in embryonic stem (ES) cells, namely Mll1 - Mll4 and Setd1a and Setd1b, thus providing an extra degree of complexity in regulating gene expression patterns. These TrxG proteins are essential for developing embryos, evident in the embryonic-lethal phenotype of Mll2 null mutants (Glaser et al., 2006). The focus of our lab is to generate homozygous multi-purpose conditional alleles in mouse ES cells for all six H3K4 methyltransferases. Using conditional mutagenesis in combination with gene expression profiling we aim to identify specific target genes of each histone methyltransferase. To assess the role of Setd1a, we disrupted the gene in embryonic stem (ES) cells and generated heterozygous mice. The breeding of heterozygous mice yielded no viable homozygous Setd1a knockout mice. Here, we will report an early embryonic lethal phenotype for Setd1a null mutants. Most homozygous embryos die in utero between days 6.5 and 7.5 early after gastrulation. Our findings indicate that Setd1a plays an essential role in mouse embryonic development.

2868/B584

Chondrogenic Activity of Novel Umbilical Cord Stem Cells (UCSCs) Is Sustained after Ex Vivo Cell Expansion.

K. Wescoe[1,2], R. Schugar[2], Y. S. Askew[2], B. M. Deasy[2,3,4]; [1]Bioengineering, University of Pittsburgh, Pittsburgh, PA, [2]Stem Cell Research Center, UPMC Children's Hopsital of Pittsburgh, Pittsburgh, PA, [3]McGowan Institute of Regenerative Medicine, Pittsburgh, PA, [4]Bioengineering and Orthopaedic Surgery, University of Pittsburgh, Pittsburgh, PA

Objective: Osteoarthritis is a growing epidemic and remains an existing clinical challenge. A novel, cell-based therapy for cartilage reconstruction is greatly needed. The umbilical cord is a source of mesenchymal stem cells (MSCs) with high expansion potential, making it an attractive cell-source for therapies requiring large numbers of cells. This project examined the chondrogenic activity of the UCSCs, and the effect of expansion on this chondrogenic potential. We tested the ability of TGF-β1 to induce chondrogenic differentiation in UCSCs at ~ 12 population doublings (PDs) and ~24 PDs. **Methods:** UC cells were isolated from male human umbilical cords, expanded to approximately 60% confluence and were used to make pellets via the standard pellet assay (2.5E5 cells/pellet). Chondrogenic induction media supplemented with either 0 or 10 ng/mL TGF-β1 was added to the pellets and changed every 3 days. After 0, 10, and 21 days of culture, pellets were harvested, paraffin embedded, sectioned, and stained with Alcian Blue and Masson's Trichrome. Average cell density at each time point was manually determined and analyzed for significant change over time using a one-way independent ANOVA a Bonferroni correction ($P< 0.05$). **Results:** Chondrogenic activity was detected in UC cell pellets in groups of both early cell age (12 PDs; young-UC) and older in vitro cell age (24 PDs, old-UC). Pellet cell density significantly decreased over 10-and 21-day culture, suggesting the cells are becoming more dispersed due to an increase in their ECM production. Pellets grown in media supplemented with TGF-β1 demonstrated increased collagen and proteoglycans content over time versus the unstimulated controls. These results were consistent for pellets made from UC cells of either early or older in vitro cell age. **Conclusion:** Stem cells derived from the umbilical cord are novel and provide benefits for cell therapy and tissue engineering. UCSCs exhibit chondrogenic activity and this differentiation capacity is not reduced with ex vivo cell expansion. Their ability to retain differentiation capacity over 21 days of culture is promising for therapies, which would require large numbers of cells.

2869/B585

An Engineered G_s-Coupled Receptor for Regulating Stem Cell Differentiation.

E. Hsiao[1,3], T. Nguyen[1], J. Ng[1], C. Manalac[1], H. Zahed Kargaran[3], R. Nissenson[2,3], B. Conklin[1,3]; [1]GICD, J. David Gladstone Institutes, San Francisco, CA, [2]Endocrine Unit, San Francisco VA Medical Center, San Francisco, CA, [3]University of California-San Francisco, San Francisco, CA

Although G-protein coupled receptors (GPCRs) are strongly implicated in development, their precise functions in differentiation have not been well defined. We previously showed that osteoblast-specific expression of an engineered GPCR ("Rs1") that activates the G_s-signaling pathway induces a dramatic increase in bone mass in mice. Our observations suggest that G_s-GPCR signaling in osteoblasts may alter mesenchymal progenitor differentiation. Here we describe an embryonic stem (ES) cell model for studying the roles of G_s-signaling in early developmental events. **Methods**: We created a new single-construct tetracycline-regulated vector for expressing Rs1 and other signaling molecules. Constructs using the EF1-α (ubiquitous) or ColI-α 2.3kb (ColI(2.3); osteoblast-specific) promoter to express mCherry and Rs1 have been introduced into mouse ES cells by directed-targeting into the Rosa26 locus. **Results**: Rs1 expression in the ES cells showed both basal signaling activity of Rs1 (9.82 +/- 0.23 *vs*. 53.4 +/- 0.82 fmol cAMP/cell) and ligand-mediated activation by the 5HT4 agonist RS67333 (348.2 +/- 10.36 fmol cAMP/cell). Teratomas made from the ES cells suggest that ColI(2.3)-driven Rs1 expression decreases cartilage glycoprotein formation in 3-week-old teratomas as determined by PAS (periodic acid Schiff) staining (2.0 +/- 1.0 *vs*. 0.5 +/- 0.4 % area of tumor, p=0.1). In contrast, the ColI(2.3)-driven Rs1 ES cell line showed larger calcified nodules than control lines in an *in vitro* osteoblast differentiation assay after 27 days of differentiation. **Conclusions**: These results suggest that ColI(2.3)-driven Rs1 expression in maturing osteoblasts decreases cartilage precursor formation in 3-week-old teratomas and increases calcified nodule size when differentiated into

osteoblasts *in vitro*. Our findings suggest that G_s signaling regulates the balance of cartilage *vs*. bone formation. Our model systems will provide a foundation for further studies on GPCR signaling in stem cell differentiation.

2870/B586

Functional Modulation of Oct-4 Activity by Pyruvate Kinase Isozyme Type M2 (PKM2) in Undifferentiated State.

J. Lee[1], H. Kim[1], Y. Han[2], J. Kim[1]; [1]Life Science, Sogang University, Seoul, South Korea, [2]Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, South Korea

The Oct-4 gene encodes a transcription factor that plays an important role in maintaining the pluripotent state of embryonic stem cells and may prevent expression of genes activated during differentiation. Although its role in maintaining embryonic stem cell pluripotency is well established, there is still little known about the binding partners that regulate its function. To identify proteins that control Oct-4 function, we used affinity chromatography on immobilized Oct-4 (POU) together with MALDI-TOF (matrix-assisted laser-desorption ionization-time-of-flight) MS (mass spectrometry), and isolated a novel Oct-4-interacting protein, pyruvate kinase type M2 (PKM2 or M2-PK). PKM2 is an isozyme of pyruvate kinase that is specifically expressed in proliferating cells, such as embryonic stem cells, embryonic carcinoma cells, as well as cancer cells. Oct-4 and PKM2 were co-affinity precipitated from cell extracts, and glutathione S-transferase pull-down assays revealed that the POU DNA binding domain of Oct-4 was required for interaction with PKM2. In addition, the C-terminal domain of PKM2 (amino acids 307-531) was involved in binding to Oct-4. Moreover, ectopic expression of PKM2 greatly enhanced Oct-4-mediated transcription. These observations indicate that the transactivation potential of the Oct-4 transcription factor is increased by PKM2.

2871/B587

Increased Production of BMP4 from STO Cells Inhibit Proliferation of Mouse Stem Cells In Vitro.

K. Kim, Y. Lee, Y. Ryu, I. Han; Biological Sciences, University of Ulsan, Ulsan, South Korea

Embryonic stem cells (ESCs) can be propagated in definitely in vitro on feeder layers of mouse STO fibroblast cells. The STO cells secrete several cytokines that are essential for ESCs to maintain their undifferentiated state. Among these cytokines, BMP4 (Bone Morphogenetic Protein 4) plays key roles in a variety of processes in embryonic development. Recent study has shown that activation of NF-κB brings about transcriptional repression of Bmp4 gene. Here we have demonstrated that the inhibition of NF-κB signaling results in the loss of feeder cell function through up-regulation of BMP4 expression. Mouse ESCs could not form colonies in vitro on the STO cells, which were transduced with dnIκB-containing adenovirus. These data reveal that the enhanced production of BMP4 from STO cell inhibit the proliferation of ESCs.

2872/B588

FGF Signaling Maintains Proliferation of Neural Progenitors through the Regulation of Cks1 Transcription.

A. J. Darr[2,3], P. Tsoulfas[1,2,3]; [1]The Department of Neurological Surgery, University of Miami Miller School of Medicine, Miami, FL, [2]The Miami Project to Cure Paralysis, University of Miami Miller School of Medicine, Miami, FL, [3]Neuroscience Program, University of Miami Miller School of Medicine, Miami Beach, FL

Identifying the mechanisms that regulate neural progenitor cell (NPC) proliferation and differentiation is important for understanding CNS development among different phyla. Our work has focused specifically on understanding how mitogens, like basic fibroblast growth factor (FGF2), regulate the proliferation of NPCs. Mitogenic factors and serum are thought to drive cell cycle and therefore proliferation mainly by activating G1 cyclin-dependent kinases (cdks). The general hypothesis that we are trying to address here is that FGF2 promotes cell cycle progression and proliferation of NPCs by facilitating the degradation of potent cell cycle inhibitors in addition to the activation of cdks. We find that NPCs harvested from E14 rat cerebral cortex in the presence of FGF2 express high protein levels of the SCFskp2 component Cks1b and undetectable levels of cell cycle inhibitor p27Kip1, while removal of FGF2 causes a sharp decrease in Cks1b and corresponding increase in p27kip1. Furthermore, our RNA expression data shows that Cks1 mRNA is reduced in non-dividing NPCs but is present in proliferating progenitors, suggesting that Cks1b is being regulated at the transcriptional level. Analysis of the putative promoter of Cks1b revealed numerous conserved transcription factor consensus sites that could potentially play a role in regulation of Cks1b transcription, including a consensus site for E2F. We used chromatin immunoprecipitation and luciferase assays to identify which E2Fs occupy and regulate the transcription of Cks1b under different conditions of mitogen stimulation. Our results suggest that E2F4 occupies the promoter of Cks1b exclusively under non-proliferating conditions, and mutation of an E2F consensus site immediately upstream of the putative transcriptional start site of Cks1b enhances the activity of a luciferase reporter gene in synchronized cells. Our data suggest that E2F4 acts as a repressor of Cks1b activity in non-proliferating NPCs and that release of E2F4 from the Cks1b promoter upon stimulation with mitogens coincides with increased Cks1b expression, a rapid decrease in p27kip1, and maintenance of proliferation of NPCs in vitro.

2873/B589

Combined Canonical and Non-Canonical Wnts Enhance the Proliferation of STRO-3 Stem Cells.

G. Xiang[1, 2], T. Cassino[1], H. Sondermeijer[2], F. See[2], T. Seki[2], S. Itescu[2]; [1]Stem Cell Research Center, Pittsburgh, PA, [2]Columbia University, New York, NY

Wnt signaling plays a critical role in cell proliferation, differentiation and cell fate decision. Canonical and non-canonical Wnt signaling pathways differentially affect the proliferation and differentiation of stem and progenitor cells. Here, we first identified the expression profile of Wnt signaling molecules in STRO-3 cells, a subpopulation of mesenchymal stem cells, and then subjected STRO-3 cells to combinations of Wnt5a and Wnt3a at different ratios to examine effects of these combinations on proliferation and differentiation toward cardiomyocytes. BrdU incorporation and MTS assay show that STRO-3 cells in the presence of combined Wnt5a and Wnt3a proliferated markedly faster relative to Wnt5a or Wnt3a or medium alone. However, when reagents capable of inducing cardiomyogenesis (5-azacytidine, DMSO and BMP4) were added to growth medium with or without the addition of Wnt5a or Wnt3a alone or combined, no transcripts of cardiac marker genes Nkx2.5, GATA4 and β-MHC were expressed. Our results suggest that cross-talk between canonical and non-canonical Wnt signaling pathways might provide a benefit to proliferation of STRO-3 cells, whereas exogenous Wnt5a, Wnt3a, and combined Wnt5a and Wnt3a are not sufficient to induce differentiation of STRO-3 cells toward cardiomyocytes.

2874/B590

The Role of CD146 in Proliferation and Differentiation of Umbilical Cord Derived Stem Cells.

R. Schugar[1,3], J. J. Nance[1], B. Schmidt[1,3], B. M. Deasy[1,2,3]; [1]Stem Cell Research Center, Children's Hospital of Pittsburgh, Pittsburgh, PA, [2]Orthopaedic Surgery and Bioengineering, University of Pittsburgh, Pittsburgh, PA, [3]McGowan Institute for Regenerative Medicine, Pittsburgh, PA

We have developed an isolation technique which consistently produces an umbilical cord stem cell (UCSC) population highly positive for several mesenchymal stem cell (MSC) -identifying markers including CD44, CD73, CD90, and CD105. Similar to other stem cell populations, we have found that the UCSCs can be heterogeneous for expression of some markers. Expression levels of MSC-identifying markers remain homogeneous, but additional surface markers, such as CD146, fluctuate in vitro (mean range, 30-59%, n≥10). These variations may signify subpopulations within the heterogeneous UCSC mix, thus we have separated the CD146+ and CD146- fractions from the CD146+/- parent population to characterize the role of CD146 in proliferative and differentiation capabilities between the phenotypically distinct groups. METHODS FACS: UCSCs were sorted into CD146+ and CD146- fractions. Image Capture: Visible light images of CD146+, CD146- and the parent populations were captured every 10 minutes over 96 hours using the Live Automated Cell Imager under normal culture conditions. Population doublings (PDs), doubling times (DTs), and cell division times were obtained using the acquired images. Osteogenic Assay: UCSCs were treated with 0 or 100ng/ml BMP4 for 5 days and the level of osteogenesis was determined by alkaline phosphatase expression. RESULTS CD146+ UCSCs underwent 5.5 PDs, whereas CD146- UC-MSCs underwent 4.6 PDs; the parent population underwent 3.9 PDs. There is no significant difference in either the PDT (hours) of the CD146+ (18.4±0.8), CD146- (19.2±2.1), and parent population (19.7±1.7), or the cell division time (14.3±1.4, 14.5±0.9, and 14.6±2.5 hours, respectively). However, the parent population and the CD146- fraction have greater alkaline phosphatase expression than the CD146+ sorted cells following BMP4 stimulation. CONCLUSIONS We have found that the UCSCs are heterogeneous for their expression of CD146; flow cytometry may be used to separate the CD146+ and CD146- fractions. While we do not detect differences related to proliferation in CD146+ and CD146- cells, these cells have been shown to differ in differentiation behaviors. Together, these results raise questions as to the role of CD146 in a stem cell population.

2875/B591

Inhibitory Effects of Phylligenin on the Proliferation of Cultured Rat Neural Stem Cells.

S. Lee[1], H. Go[1], C. Choi[1], S. Rhee[1], C. Shin[2], K. Ko[1]; [1]Pharmacology, Seoul National University, Seoul, South Korea, [2]Pharmacology, Konkuk University, Seoul, South Korea

Neural stem cells (NSC) differentiate into astrocyte, neuron and oligodendrocytes, which is controlled by many factors in brain. Recent evidence suggests that small molecules including natural products may modulate the proliferation and differentiation of NSC. Phylligenin is a lignan that is isolated from the fruits of Forsythia koreana, with reported anti-inflammatory activity. We investigated effects of phylligenin on NSC proliferation and differentiation. The treatment of phylligenin decreased the number of proliferating NSC in culture, with only marginal effects. if any, to differentiated cell types such as neuron and astrocyte. To examine the mechanism of the decreased NSC number, we used FACS analysis and it showed increased apoptosis and decreased proliferation of NSC after phylligenin treatment. We also found that proliferation of other cell lines such as C6 and U87MG glioma cells, was inhibited by phylligenin. These results suggest that phylligenin have selective toxicity toward rapidly proliferating cells such as neural stem cells, which might be useful in the treatment of brain cancers focusing on the inhibitory effects on cancer stem cell.

2876/B592

Osteopontin Is a Critical Factor for Natural Killer Cell Development in the Bone Marrow Microenvironment.

J. Chung, S. Yoon, M. Kim, I. Choi; Stem Cell Research Center, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, South Korea

Natural killer (NK) cells are bone-marrow-derived lymphocytes that share a common progenitor with T cells. NK cells constitute an essential element of the innate immune response, while the majority of B and T lymphocytes take part in adaptive immune response. Although the model of NK development from hematopoietic stem cells (HSC) has been previously proposed, the factors and the detailed mechanisms that drive NK differentiation from HSC still remain to be elucidated. Here, we performed SAGE (Sequential Analysis of Gene Expression) screening to isolate the genes that stage-specifically expressed during NK differentiation, and identified osteopontin (OPN) as a key factor in NK development. OPN-deficient mice exhibited impairments of NK development in a variety of tissues in which the expressions of CD122 and NK cell receptors were reduced, and the expression of OPN was induced by IL-15 in bone marrow stromal cells. Accordingly, the OPN-deficient mice showed reduced capability of tumor rejection. In addition, OPN upregulated the expression of T-bet during NK differentiation, and OPN-driven NK maturation was suppressed in T-bet-deficient hematopoietic precursor cells. Collectively, these results show that paracrine OPN signaling is crucial for CD122 expression and NK cell maturation, and performs an important function in anti-tumor surveillance.

2877/B593

Microrna-125b Promotes Neuronal Differentiation in SH-SY5Y Cells by Repressing Targeted Nonneuronal Transcripts.

M. T. Le[1,3], H. Xie[1,2], B. Zhou[2], H. Yang[4], B. Lim[3], H. F. Lodish[2]; [1]Computation and Systems Biology, Singapore-MIT Alliance, Singapore, [2]Whitehead Institute for Biomedical Research, Cambridge, MA, [3]Stem Cell and Developmental Biology, Genome Institute of Singapore, Singapore, [4]Institute of Bioinformatics, Singapore

MicroRNAs are a class of small non-coding RNAs that regulate gene expression at the post-transcriptional level. Research on miRNAs has highlighted the importance of this pathway in every aspect of neural development. However, the specific functions of many neural-enriched miRNAs are poorly understood. We report here the expression profile of microRNAs in neuronal differentiation of the human neuroblastoma cell line SH-SY5Y. Six microRNAs were significantly upregulated following the sequential treatments of SH-SY5Y cells with all-trans¬-retinoic acid and brain-derived neurotrophic factor. Using a quantitative neurite outgrowth assay, we demonstrate that overexpression of mir-124a or mir-125b enhances the percentage of differentiated SH-SY5Y cells with neurite outgrowth. Subsequently, we focused our functional

analysis on mir-125b. Neurite outgrowth assay and quantification of neuronal marker expression elucidate that mir-125b plays an important role in both spontaneous and induced differentiation of SH-SH5Y cells. Interestingly, mir-125b is also upregulated in the human neural progenitor ReNcell VM cells during differentiation; overexpression of mir-125b significantly promotes neurite outgrowth of these cells. To identify the downstream effectors of mir-125b, we profiled the global changes in gene expression following the overexpression and knockdown of mir-125b in SH-SY5Y cells. Remarkably, overexpression of mir-125b represses the expression of its 97 computationally predicted targets, most of which are non-neuronal transcripts, thereby driving the gene expression profile of SH-SY5Y cells towards a neuronal transcriptome. Together, we report here for the first time the important role of mir-125b in differentiation of neuronal cells.

2879/B595

Different Mechanisms of Liver Recovery Are Studied with Carbon Tetrachloride-Induced and hepatectomized mice.

Y. Kwon, M. Seo, Y. Lee, S. Choi, M. Bae, S. Choi, E. Lee, D. Kim, Y. Hwang; Division of Biological Science, Gachon Univ. of Medicine & Science, Incheon, South Korea

Liver has a strong ability of recovery after hepatic injury or resection. It has been reported that HSPs (Heat shock protein) are expressed during liver regeneration after being damaged. We already confirm it using micro array analysis and western blot. However, the mechanisms of liver regeneration are not fully established. It has been uncertain which factors make differences between chemical and physical damages, and it has not been understood what the origin of hepatocyte during liver recovery. The potential origin of hepatocytes was examined with HGF (Hepatocyte growth factor) , HNF-1(Hepatocyte nuclear factor, a nuclear marker of liver epithelial, non-hematopoietic phenotype) and CD34, c-kit, AFP(Alpha-fetoprotein), and Actin in female mice. We did experiments using Caveolin-1 antibody that gave us the reports of cell proliferation in the liver. The expressions of these genes in Carbon tetrachloride (CCl4)-administered and hepatectomized mice livers were performed at various time points to establish gene expression profile during injury and regeneration stages. We used 4weeks ICR mice and divided into 4 groups given CCl4 by IP injection twice a week for 2weeks; the liver samples were obtained after 6hr, 24hr, 48hr, 2weeks. We had injected CCl4 for a single dose of 1ml/kg diluted with mineral oil and the total volume is 200ul. Partial hepatectomy was 70% resection of adult liver and the samples were provided from hepatectomy groups 2days, 8days after operation. RT-PCR was performed to check mRNA expression and western blot was prosecuted for detecting protein. We confirmed that there were different mechanisms during recovery between CCl4-administered and hepatectomy groups. It suggests that clustering analysis of expression patterns of several genes give insights into mechanism of liver recovery. It would be established that the specific origin of hepatocytes in different recovery process is reported through further studies. This work was supported by Kopri grant PE08060.

2880/B596

Mature Adipocyte-Derived Dedifferentiated Fat Cells Exhibit Multilineage Potential.

K. Kano[1], Y. Oki[1], T. Kazama[1], H. Nobusue[1], T. Matsumoto[2]; [1]Laboratory of Cell and Tissue Biology, College of Bioresource Sciences, Nihon University, Fujisawa, Japan, [2]Division of Cell Regeneration and Transplantation, Advanced Medical Research Center, School of Medicine, Nihon University, Tokyo, Japan

When mature adipocytes are subjected to an in vitro dedifferentiation strategy referred to as ceiling culture, these mature adipocytes can revert to a more primitive phenotype and gain cell proliferative ability. We refer to these cells as dedifferentiated fat (DFAT) cells. In the present study, we examined the multilineage differentiation potential of DFAT cells. DFAT cells obtained from adipose tissues of various animals exhibited a fibroblast-like morphology and sustained high proliferative activity. Flow cytometric analysis revealed that DFAT cells comprised a highly homogeneous cell population compared with that of adipose-derived stem/stromal cells (ASCs), although then cell-surface antigen profile of DFAT cells was very similar to that of ASCs. DFAT cells lost expression of mature adipocytes marker genes but retained or gained expression of mesenchymal lineage-committed marker genes such as peroxisome proliferator-activated receptor gamma (PPARg), RUNX2, and SOX9. In vitro differentiation analysis revealed that DFAT cells could differentiate into adipocytes, chondrocytes, and osteoblasts under appropriate culture conditions. DFAT cells also formed osteoid matrix when implanted subcutaneously into nude mice. In addition, clonally expanded porcine DFAT cells showed the ability to differentiate into multiple mesenchymal cell lineages. These results indicate that DFAT cells represent a type of multipotent progenitor cell.

2881/B597

The Competency of Mesodermal Cells to Give Rise to Myotome Fibers in Xenopus Laevis.

V. Krneta-Stankic, C. Domingo; San Francisco State University, San Francisco, CA

Somites are blocks of mesodermal tissue that give rise to the vertebral column and segmented musculature of the vertebrate adult. Somitogenesis consists of the periodic formation of somites on either side of the neural tube that proceeds in anterior to posterior progression. Disruption of somitogenesis is associated with congenital defects and spinal deformities. The signals that pattern somites along the anterior-posterior (AP) axis remain poorly understood. We examined the competency of mesodermal cells to form somites consisting of aligned myotome fibers. Fate mapping experiments show that at the onset of gastrulation, mesodermal cells dorsal lateral (DL) to the prospective notochord and the lower lip (LL) cells give rise to cells positioned within the presomitic mesoderm, which subsequently segments to form somites consisting of aligned myotome fibers. Given that gastrula cells are fated to form myotome fibers, we asked whether DLL and LL cells are competent to form myotome fibers at these early stages. We transplanted DLL and LL cells (stage 11) to the somites in the anterior region of a tailbud-stage embryo. We observed that the majority of these gastrula cells failed to form myotome fibers. In contrast, cells grafted from the PSM region of older stage embryos (stage 18) to the somites in the anterior region of tailbud-stage embryos were able to form elongated and aligned myotome fibers. Additional transplantation experiments showed that mesodermal cells surrounding the blastopore region gradually become more competent to form myotome fibers over time. These results suggest that gastrula mesoderm cells require additional signals that are present at later stages of somitogenesis within the PSM that allow cells to become competent to form properly aligned myotome fibers. We hypothesize that signals associated with preparing the PSM to undergo segmentation may also be involved in instructing these cells to form myotome fibers.

Cell Culture (2882 – 2903)

2882/B599

A Mitogenic and Anti-Proliferative Lectin from Arisaema Utile.

V. Dhuna, J. Singh, S. Kamboj; Molecular Biology and Biochemistry, Guru Nanak Dev University, Amritsar, India

Objective: Lectins are defined as carbohydrate binding proteins other that enzymes and antibodies. Lectins have emerged as very important macromolecular tools to recognize carbohydrates on cell surfaces. The present work is designed to purify and characterize monocot lectins with interesting biological properties from Indian monocot plants. Methods: On the basis of sugar specificity determined by hemagglutination, asialofetuin-linked affinity was used to purify monocot lectins. Mitogenic, anti-fungal and anti-proliferative potential were determined through MTT, anti-fungal and sulphorhodamine-B assays respectively. Results: Arisaema utile lectin (AUL) gave a single band in SDS-PAGE at pH 8.3 corresponding to subunit Mr 13.5 kDa. The native molecular mass of 54 kDa suggested a homotetrameric structure. Like other monocot lectins, AUL gave multiple bands in isoelectric focusing and in native PAGE at pH 8.3. AUL was inhibited by N-acetyl-D-lactosamine (LacNAc), a disaccharide and asialofetuin, a complex desialylated serum glycoprotein. Amino acid modification studies of AUL revealed the involvement of tryptophan and tyrosine residues involved in lectin-sugar interaction. The lectin showed potent mitogenic response towards human lymphocytes. The mitogenic activity of AUL was even more than Con A, a standard well-known plant mitogen. AUL exhibited a fluorescence emission maximum (λmax) at 340 nm upon excitation at 295 nm. Using Far UV CD spectra the estimated secondary structure was 37% α-helix, 25% β-sheet and 38% random contributions. In vitro anti-proliferative activity of AUL was tested on eleven different human cancer cell lines viz. MCF-7 (Breast), SK-N-SH (CNS), 502713 (Colon), Colo-205 (Colon), HCT-15 (Colon), HT-29 (Colon), SW-620 (Colon), Hep-2 (Liver), IMR-32 (Neuroblastoma), DU-145 (Prostate) and PC-3 (Prostate). The concentrations of AUL which produced 50% inhibition (IC50) of cancer cell lines viz. SW-620, HCT-15, SK-N-SH, IMR-32, Colo-205 and HT-29 at 38, 42, 43, 49, 50 and 89 µg/ml. respectively. Conclusion: The purified Arisaema utile lectin was found mitogenic on human lymphocytes and anti-proliferative on human cancer cell lines.

2883/B600

Endoglin Haploinsufficiency Modifies Cell Funtions in Dermal Fibroblasts in a TGFβ Independent Manner.

M. Pericacho, S. Velasco, M. Prieto, J. M. López-Novoa, A. Rodriguez-Barbero; Fisiología y Farmacología, Universidad de Salamanca, Salamanca, Spain

Endoglin is a transforming growth factor beta (TGFβ) co-receptor involved in vascular remodeling, angiogenesis and fibrosis. In most cell types, TGFβ binds to the ubiquitously expressed TGFβ receptor type I, also known as activin-like kinase receptor 5 (ALK5), that activates Smad2 and Smad3. In a few other cell types, TGFβ activates ALK1, in the presence of functional ALK5, resulting in phosphorylation of Smad1 and Smad5. It has been proposed that, in endothelial cells, ALK1 activation triggers proliferation and migration, whereas ALK5 activation has the opposite effects; and that endoglin expression promotes ALK1 signaling. As endoglin is expressed mainly in endothelial cells, most of the endoglin studies has been carry out on endothelial cells, however endoglin is expressed in many other cell types, but little is known about its function in those cells. The aim of the present study was to evaluate the role of endoglin on fibroblast functions and its possible role on modulating TGFβ signalling. To this purpose we have generated primary cultures of murine dermal fibroblasts from endoglin heterozygous (Eng+/-) mice and its controls (Eng+/+). Analysis by western blot of these cells revealed that endoglin expression was reduced by 50% in Eng+/- fibroblasts. The rate of cell growth, analyzed by cell cycle studies, PCNA expression and crystal violet assays, was faster in Eng+/- fibroblasts than in control cells. In the same way, migration was observed in Eng+/- cells measured by a wound healing and a transwell system. In endothelial models shown by other authors, Eng+/- cells have deficient migration and proliferation. Whereby our data reveal the importance of endoglin in modulating proliferation and migration in dermal fibroblast, and could show a cell-type-dependent role of endoglin. Western blot analysis showed that both ALK1 and ALK5 TGFβ receptors are present in dermal fibroblast and they are functional as TGFβ1 induced Smads activation. Although no different Smad activation profile was observed between Eng+/- and Eng+/+ fibroblasts. These result suggest that the observed differences between Eng+/+ and Eng+/- could not been due to differential activation of TGFβ signalling routes.

2884/B601

Cold-Adapted Recombinant Chinese Hamster Ovary Cells Exhibit Increased Cellular Fragility and a Compromised Vimentin Intermediate Filament Network.

K. Sunley, T. Tharmalingam, M. Butler; Department of Microbiology, University of Manitoba, Winnipeg, MB, Canada

The culturing of recombinant Chinese hamster ovary cells under mild hypothermic (30°C - 33°C) conditions is known to increase cell-specific heterologous protein expression by several fold. However, such low temperature cultivation also greatly reduces the rate of cell growth, limiting the application of the high protein expression rates which occur at lower temperatures. We have previously reported the isolation of a recombinant cell line capable of enhanced rates of hypothermic growth which maintains the cold-induced elevated rates of recombinant protein expression[1]. Apparent in the population of cold-adapted cells was a dramatically increased sensitivity to mechanical stress. This is demonstrated by the inability of the adapted cells to grow within agitated suspension cultures without the aid of an extra-cellular support structure, such as a macroporous microcarrier. We have further characterized this cellular fragility in terms of their susceptibility to deformation when subjected to centrifugal force. The subset of cells within a non-adapted population which was able to resist significant deformation was completely absent in the cold-adapted line, with all measured cells compressed to a minimum of 50% of their initial height at 1700*g*. This coincides with evidence that the low temperature population appears to lack an organized vimentin filament network, compromised by intracellular proteolytic cleavage of vimentin subunits reminiscent of early apoptotic caspase activity. <hr> 1. Sunley et al. 2008. CHO Cells Adapted to Hypothermic Growth Produce High Yields of Recombinant β-Interferon. *Biotechnol. Prog.* DOI 10.1021/bp.9

2885/B602

Expression of Heat Shock Proteins (HSPs) of Mouse Cell during Hypothermic Preservation.

S. Choi, Y. Lee, M. Seo, Y. Kwon, S. Choi, M. Bae, E. Lee, D. Kim, Y. Hwang; Division of Biological Science, Gachon Univ. of Medicine & Science, Incheon, South Korea

Heat shock proteins (HSPs) are stress-inducible molecular chaperones that range in size from 12 to 43 kDa and include the α-crystallins since these proteins share many of the same physical and functional properties of other HSPs and their expression is enhanced by stress. These proteins are essential for cellular viability following cellular stress such as elevated temperature. While heat shock is known to influence the expression of many HSPs and molecular chaperones, there is little information on the influence of low temperatures on their expression. However, recent studies with heat-stressed fruits have shown a correlation between the accumulation of HSPs and the acquisition of chilling tolerance. The results suggest that HSPs could be involved to some extent in the ability of heat shock to increase chilling tolerance. So, we tried to integrate these HSPs to hypothermic preservation. Hypothermic preservation is preserving method that is used widely to preserve graft organs or cells in low temperature. Antifreeze proteins (AFPs) and some sugars are added to preserving extender, the University of Wisconsin (UW) solution. We used 5 types of cells; HepG2 cell line as a cancerous hepatocyte sample, 293T cell line as a normal kidney cell sample, isolated hepatocyte, isolated lung cell, and blood cells from adult mice. After one-day-incubation under condition of 37 C and 5% CO2, hypothermic preservation at 4C experiments were performed for 24-72 hours. Cells were divided into 9 groups by preservation solution; DMEM media, D-PBS, UW solution, UW solution with sugars (fucoidan, fucose, mannose, and xylose respectively) or AFP, and DMEM media with DMSO. We analyzed HSPs expression in protein extracted from cells by western blotting. The result of western blotting, the expression of HSPs showed us apparent change on samples. It can therefore be suggested that constitutive expression of elevated levels of HSPs might be an alternative strategy for protection against chilling injury. This work was supported by Kopri grant PE08060

2886/B603

Expression of Caveolae of Mammalian Cell during Hypothermic Preservation.

Y. Lee, S. Choi, M. Seo, Y. Kwon, E. Lee, S. Choi, M. Bae, Y. Hwang, D. Kim; Division of Biological Science, Gachon Univ. of Medicine & Science, Incheon, South Korea

During cryopreservation, freezing led to mechanical damages due to the formation of ice crystals and induced solute concentration in the fluid surrounding the ice and it caused an osmotic flux of water into the freezing compartment.Extracellular freezing caused an osmotic efflux of water from the cells, which subsequently shrink. It has been reported that HSPs (heat shock protein) were expressed and increased cell viability, but it was not fully established. In this investigation we evaluated the relationship between the expression of caveolin-1, HSP (Heat shock protein) by western-blot in mammalian cell under conditions of various extender. Caveolae are small, functionally important membrane invaginations found on the surface of many different cell types. So we supposed that cold and osmotic stress caused similar oxidative damages to the cell components and the expression of caveolin-1 was related to the condition of membrane in environmental change such as rapid freezing and thawing. Thus, caveolin-1 was probably induced through a separate signal transduction pathway. We used 5 types of cells HepG2, NCI-H292, HFT cell line. After one-day-incubation under condition of 37 C and 5% CO2, cryopreservation at 4 C experiments were performed for 24 2days. Cells were divided into 6 groups by preservation solution; RPMI media, D-PBS, UW solution, UW solution with sugars (fucoidan, fucose, mannose). We analyzed the expression of caveolin-1, HSP27 (small heat shock protein), Sep2 in proteins extracted from the cells written above by western blotting. Adding to this data, it could be analyzed by ORT (osmotic resistance test) that showed the interaction of the effect on cell viability and osmotic resistance. It suggests that the regulation of osmolarity is the important factor to maintain the cellular function under the cold shock. It would be established more specific effect of caveolin-1 on condition of freezing through further studies. This work was supported by Kopri grant PE08060.

2887/B604

Stress Induction of Novel Cytoplasmic Rods and Rings in Cancer and Primary Cells.

W. C. Carcamo[1], H. Kasahara[2], M. Satoh[3,4], J. Wilhelm[5], G. Covini[6], C. A. von Mühlen[7], E. K. Chan[1]; [1]Oral Biology, University of Florida, Gainesville, FL, [2]Physiology & Functional Genomics, University of Florida, Gainesville, FL, [3]Medicine, University of Florida, Gainesville, FL, [4]Pathology, Immunology, and Laboratory Medicine, University of Florida, Gainesville, FL, [5]Section on Cell and Developmental Biology, University of California, San Diego, La Jolla, CA, [6]Gastroenterology, Istituto Clinico Humanitas, Rozzano, Italy, [7]Rheuma Clinic, Porto Alegre, Brazil

Our laboratory has identified novel human autoantibodies recognizing conserved rods and rings (RR) in the cytoplasm of several cancer cell lines. This study aimed to characterize conditions for the formation of these RR structures. Using indirect immunofluorescence, HEp-2 cells showed one or two distinct cytoplasmic rods (~3-10 μm in length) and/or rings (2-5 μm dia.) in >95% of cells. These data were validated using newly acquired HEp-2 cells from American Tissue Culture Collection to rule out artifacts from continuing culturing and potential contamination. Simple costaining experiments have ruled out these RR from the Golgi complex, and other known cytoplasmic organelles including arsenite-induced stress granules. The number and size of the RR depend on the cell type, while some cells express 1-2 long rods others express many little rods. RR formation was independent of the cell cycle but depended on cell proliferation states and time after cell plating. RR were often detected in cells freshly replated from liquid nitrogen storage. However, after several passages, the number of RR decreased significantly but could be increased again using repeated rounds of cell trypsinization. A candidate 55kDa RR protein was identified as cytidine triphosphate synthetase (CTPS) and further demonstrated in that transfected EGFP-CTPS localized to RR. There are apparent conversion between rods and rings. A glutamine analog CTPS inhibitor 6-diazo-5-oxo-L-norleucine (DON) was shown to induce RR formation in aged HEp-2 cells after 24hrs. HEp-2 cells treated with DON had RR in ~95% of cells compared to untreated with only ~5-10%. Analyses at multiple time points showed that 24 hours was required for the formation of mature RR; however, partial RR could be detected in as early as 1 hr. Other cell lines shown positive for RR included rat NRK, mouse 3T3, and human THP-1. Our data demonstrated that similar RR can be induced in >50% of primary mouse cardiomyocytes. Thus, RR could be induced from a stress response-like mechanism in proliferating cells using a CTPS inhibitor that altered nucleotide metabolism.

2888/B605

Comparative Study of Constitutive 70 kDa Heat Shock Protein Content in the Cells of Some Amoeboid Protists.

Y. Podlipaeva[1], I. Dykova[2], L. Shmakova[3], A. Goodkov[1]; [1]Institute of Cytology, Russian Academy of Sci., St.Petersburg, Russian Federation, [2]Institute of Parasitology Academy of Sciences of Czech Republic, Ceske Budejovice, Czech Republic, [3]Institute of Physicochemical and Biological Problems in Soil Science, RAS, Moscow Region, Pushchino, Russian Federation

Constitutive level of 70 kDa family heat shock protein (HSP70) was compared in wide spectrum of naked amoebae strains, belonging to phylogenetically long distant genera - Amoeba (order Amoebida) and Acanthamoeba (order Acanthamoebida). We examined 15 strains of free living freshwater Amoeba species (The Collection of Strains of Free-living Freshwater Amoebae, Laboratory of Cytology of Unicellular Organisms, Institute of Cytology RAS), 7 strains of amphizoic Acanthamoeba species (The Collection of Cultures, Department of Eukaryotic Microorganisms Infecting Fish, Parasitological Institute, CAS), and one strain of free living Acanthamoeba sp. isolated from the East-Siberian tundra soils. Method of immunoblotting was applied. It appeared that in all species of genus Amoeba, independently of their origin, the level of constitutive HSP70 was more or less pronounced. Noticeable level of constitutive HSP70 was found in 2 of 7 amphizoic acanthamoebae strains, being rather high in the first strain and hardly observed in the second one. In tundra strain of Acanthamoeba high level of constitutive HSP70 was detected. So, we still failed to reveal any sort of correlation between the constitutive HSP70 content and biology characters of studied unicellular organisms. The zone stained with monoclonal anti-HSP70 antibodies in all free living Amoeba and amphizoic Acanthamoeba strains corresponded to the position of about 70 kDa MW, whereas this zone in tundra soil Acanthamoeba was localized in the position of about 60 kDa, quite similar to that of ancient strain of Acanthamoeba sp. isolated from the permafrost soil samples, as we have described earlier (Abstr. Am. Soc. Cell Biol. 46th Ann. Meet. 2006).

2889/B606

Establishment of Mouse Osteoblastic Cell Lines (MOBs) Using Human Telomerase Reverse Transcriptase (hTERT).

M. M. Thi[1], M. Urban-Maldonado[1], S. O. Suadicani[2,1], D. C. Spray[1]; [1]Dominick P. Purpura Department of Neuroscience, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, [2]Department of Urology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY

Primary osteoblasts, like many other primary cells, reach replicative senescence after a few passages. In order to overcome this problem, previous studies have isolated cells from osteosarcomas or immortalized osteoblasts from different origins using viral genes such as Simian virus 40 (SV40) T antigen. Although these immortalization procedures are reliable, immortalized cells may lose the phenotypic properties of primary osteoblasts and could become genetically unstable. To establish a osteoblast cell line that would provide uniform cell populations for functional and long-term gene expression studies, we immortalized neonatal mouse calvarial osteoblasts from C57BL/6J mice using human telomerase reverse transcriptase (hTERT). Four mouse osteoblastic cell lines expressing hTERT (MOB A-D) were generated from individual neonatal mice with the MOB-C line being most similar morphologically to primary osteoblasts (PMOBs). RT-PCR demonstrated that MOBs abundantly expressed osteoblastic markers [osteopontin, Type I collagen, periostin (OSF-2), and parathyroid hormone receptor (PTHR)]. Similar to PMOBs, MOB-B and -C expressed higher levels of osteocalcin than MOB-A and -D. MOB-A, -B and -C produced moderate to high levels of alkaline phosphatase expression, whereas MOB-D produced little or no expression. Alkaline phosphatase expression was detected in MOB-B and -C as early as 3 days after seeding at density of 10^4 cells/cm^2. Matrix mineralization occured markedly in MOB-C, moderately in MOB-B and barely in MOB-A, as indicated by von Kossa staining. Furthermore, all MOB-A, -B, and -C cell lines consistently maintained their phenotypic markers up to 30 population doublings. These findings demonstrate establishment of new and very stable osteoblastic cell lines from mice: MOB-A (preosteoblastic), MOB-B and MOB-C (osteoblastic). Support: NIH F32HL082130, AHA 0735377N, NS 041282.

2890/B607

Calcification on Cultured Human Dental Pulp Cells Exposed to High Glucose Level.

T. Okamura, T. Nishikawa, A. Tanaka; Oral Pathology, Osaka Dental University, Hirakata, Japan

[Objective]Undifferentiated α-SMA-positive dental pulp cells have been considered to be progenitors of odontoblasts. α-SMA is also expressed by vascular smooth muscle cells, which are well-known to differentiate to osteoblasts. This osteogenesis is up-regulated under hyperglycemic condition. Proliferation and differentiation of dental pulp cells are also considered up-regulated under high-glucose condition. By the method of detailed examination of dental pulp cell responses to high and normal glucose concentrations, the present study was designed to obtain more information about dental pulp cells, odontoblast progenitors and odontogenesis.[Methods]Both cell proliferation and differentiation of human dental pulp cells were examined. Differentiation was confirmed by examining ALP activity and calcified nodules as well as gene expressions of osteocalcin and dentin sialoprotein. Mural cell markers, eNOS, NG2, calponin and α-SMA were immunostained and quantitatively evaluated. The data obtained using high-glucose media were compared with those obtained using normal media. [Results]Both proliferation and differentiation of human dental pulp-derived cells were up-regulated in hig-hglucose media. [Conclusions]NG2 and calponin are more suitable markers for odontoblast progenitors than α-SMA. Odontogenesis is different from osteogenesis and may be regulated by endothelial cells.

2891/B608

Obtaining and Characterization of a Population of Progenitor Cells in Corneal Stroma.

S. Del Olmo Aguado[1], M. Martinez-Garcia[1,2], J. Merayo-Lloves[1], I. Alcalde[3,2,1], P. Gallego-Muñoz[1], R. Cantalapiedra[1]; [1]IOBA, University of Valladolid, Valladolid, Spain, [2]Dpt. Cell Biology and Histology, University of Valladolid, Valladolid, Spain, [3]INCYL, University of Valladolid, Valladolid, Spain

Objective: The establishment of cell culture from corneal stroma in order to detect the presence of stem cells in the limbus and the central cornea, without epithelial stimulus. Methods: Cells were obtained from the disintegration of the matrix of corneal stroma of hens in two areas (limbus and centre). They were maintained in culture for their characterization with inmunocytochemistry techniques and to test their proliferation ability. Human de-epithelialized corneoscleral ring culture was used as control for the markers. Results: Cells with positive

reactivity to Oct-4, Vimentin, Thy, Pax-6 were obtained. Oct-4 appeared in a 3.105% in limbus and in a 0.609% in the central cornea cultures. Pax-6 appeared in a 3.02% in limbus and in a 1.04% in the central cornea cultures. This cellular line remained stable in its multiplication ability all along the study (10 subcultures). Conclusions: A cellular line from corneal stroma cells was obtained. This line remains proliferative and with a high viability; it shows positive markers of mesenchymal stem cells, mainly located in the limbal stroma of the cornea.

2892/B609

Isolation, Characterization, and Immunogenicity of Human Umbilical Cord-Derived Mesenchymal Stem Cells.

K. Kita[1,2], G. G. Gauglitz[1,2,3], C. Lo[4], D. Y. Medina[5], S. Williamson[2], R. A. Cox[2,6], L. A. Vergara[7], D. N. Herndon[1,2], M. G. Jeschke[1,2,8]; [1]Department of Surgery, The University of Texas Medical Branch, Galveston, TX, [2]Shriners Hospitals for Children, The University of Texas Medical Branch, Galveston, TX, [3]Department of Dermatology and Allergology, Ludwig Maximilians University, Munich, Germany, [4]Medical Student Summer Research Program, School of Medicine, The University of Texas Medical Branch, Galveston, TX, [5]Summer Undergraduate Research Program and Department of Entomology, Texas A&M University, College Station, TX, [6]Department of Pathology, The University of Texas Medical Branch, Galveston, TX, [7]Optical Imaging Laboratory, Department of Neuroscience and Cell Biology, The University of Texas Medical Branch, Galveston, TX, [8]Department of Biochemistry and Molecular Biology, The University of Texas Medical Branch, Galveston, TX

The use of human stem cells (SCs) is a promising approach for the treatment of many diseases and injuries. Umbilical cord/amnion membrane represents a good source for SCs, because those tissues are an abundant source and there are less ethical issues unlike embryonic SCs. We aimed to isolate and characterize adult SCs from umbilical cord/amnion membrane by flow cytometry, immunofluorescence microscopy, Western blotting, and RT-PCR. In order to approach clinical use, we also proposed to culture isolated SCs onto clinically used supporting membranes. Ten to 14 days after starting the culture, we observed significant amounts of cells migrating from implants. The majority of cells were mesenchymal (fibroblastic) cells, but we also observed sub-population of epithelial-like cells. Because mesenchymal stem cells (MSCs) are thought to show less immunogenicity, we first focused on characterization of MSCs. Cell surface expression of MSC markers (CD73 and CD105) was confirmed by flow cytometry. Flow cytometry or immunostaining also demonstrated significant expression of typical SC-specific markers, such as SSEA-4, Oct-4 and Nanog. MSCs did not lose the expression of Oct-4 and Nanog after freeze-thawing. MSCs could differentiate into at least adipogenic and osteogenic lineages. On the other hand, typical embryonic SC-specific markers (FGF-4, Sox-2, Rex-1, telomerase reverse transcriptase), were not expressed. Thus, MSCs we isolated are distinct from embryonic SCs. As reported in embryonic SCs, our SCs do not express major histocompatible complex (MHC) class II, which is thought to have a greater effect than other MHC molecules during immunological rejection by a host. Although mRNA expression of MHC class II (HLA-DR) was slightly induced by inflammatory cytokine, interferon-γ, the change of cell surface MHC class II level was subtle. This result suggests that our MSCs cause less immune response by a host after transplantation. MSCs showed healthy growth on clinically used supporting materials. We thus believe that SCs isolated by our method maintain typical characteristics of MSC in vitro, and may be an attractive source for biomaterials including "customized skin" to provide improved care of patients.

2893/B610

Self-Renewal, Maintenance, and Characterization of Adult Stem Cells on GEM TM Beads (Global Cell Solutions).

V. P. Pearce[1], J. Shankardas[1], A. A. Sule[2,3], B. Justice[4], D. Dimitrijevich[5]; [1]Pharmacology and Neuroscience, University of North Texas Health Science Center at Fort Worth, Fort Worth, TX, [2]Integrative Physiology, University of North Texas Health Science Center at Fort Worth, Fort Worth, TX, [3]Graduate School of Biomedical Sciences, University of North Texas Health Science Center at Fort Worth, Fort Worth, TX, [4]Global Cell Solutions, Charlottesville, VA, [5]Integrative Physiology, University of North Texas Health Science Center at Fort Worth, Fort Worth, TX

Background: During homeostasis resident adult stem cells self-renew, and generate differentiation competent progeny. However, circulating stem cells appear to display considerable plasticity that is critical in repair. The roles of both in aging and cancer are poorly understood. We are interested in using self-renewal capabilities to investigate propagation in 3D and monolayer cultures and determine conditions that would retain "stemness", or promote selection of specific linage subtypes. Purpose: To determine if human corneal epithelial and neural stem cells expand on the alginate GEMTM beads and be utilized for further cellular and molecular studies of self-renewal and plasticity. Methods: Corneal epithelial adult stem cells, isolated from human donor corneas, were incubated with the GEMTM with gentle agitation per manufacturer's instructions. Similarly, neural stem cells isolated from fetal tissue (ReNcell NSC, Chemicon) were incubated without agitation. Cells attached to GEMTM were subcultured on collagen type IV, laminin and tenacin C coated surfaces. Viability was established by neutral red staining. Cytoskeletal protein (beta-actin) expression was determined by immunofluoresence on fixed GEMTM bearing cells cultured in suspension and in Rotating Wall Vessel. Results: Corneal epithelial and neural stem cells adhere, grow, and remain viable on GEMTM. Corneal stem cells form large aggregates of cells with GEMs, while neural stem cells form neurospheres. The protein expression in cells on the GEMTM may be compared with those in monolayers by immunofluorescence. The cells from the GEM can be subcultured onto collagen IV, laminin and tenacin C. Culture in the RWV formed of tissue like aggregates on composed of GEMTM substrate and cells. Conclusions: Corneal epithelial stem cells can be cultured and maintained on the GEMTM substrate and expanded for use in downstream experiments without trypsinization. With modification of the GEMTM, neural stem cells can also be cultured and maintained on GEMTM. The protein expression in cells on GEMTM beads and their fate can be determined by indirect immunofluorescence.

2894/B611

Establishment of a Preadipocyte Cell Line Derived from Mature Adipocytes of GFP Transgenic Mice and Formation of Adipose Tissue.

H. Nobusue, K. Kano; Division of Applied Life Science, Graduate School of Bioresource Sciences Nihon University, Fujisawa, Japan

We established a preadipocyte cell line from mature adipocytes obtained from subcutaneous fat tissue of green fluorescent protein (GFP) transgenic mice. The floating top layer, containing mature adipocytes, was isolated from the subcutaneous fat tissue by collagenase digestion

and filtration. Fluorescence-activated cell sorting (FACS) and microscopic analysis revealed that the floating cell fraction comprised of a highly homogeneous adipocyte population without any adipose stromal-vascular cells. Isolated mature adipocytes dedifferentiated into fibroblast-like cells and actively proliferated in ceiling culture. In vitro studies showed that the cells could redifferentiate into mature adipocytes in an identical way as 3T3-L1 preadipocytes. No changes in the differentiation pattern were observed during the propagation of these cells. They have been successfully maintained and differentiated for at least 22 passages. We have named these cells dedifferentiated fat (DFAT-GFP) cells. When DFAT-GFP cells were implanted subcutaneously in C57BL/6N mice, they developed highly vascularized fat pads that morphologically resembled normal subcutaneous adipose tissue and consisted of GFP-positive cells; however, implanted 3T3-L1 cells did not have such an effect on the mice. We conclude that DFAT-GFP cells could provide a model that would enable us to study the mechanisms of adipocyte differentiation and adipose tissue formation in vivo and in vitro, respectively.

2895/B612

Pluronic F-68 as a Viability Enhancer in Feeder-free Production of Red Blood Cells.

E. Baek, H. Kim, J. Kim, H. Kim; Laboratory Medicine, Yonsei Univ. College of Medicine, Seoul, South Korea

Background; As the shortage of donated red blood cells (RBCs) became worse, generation of RBCs in vitro from hematopoietic stem cells became of outstanding importance. However, the viability of erythroid cells are decreased as maturation proceeds, urging development of new reagents which could enhance RBC production. Pluronic F-68 (PF) is a nonionic block copolymer chemical surfactant known to be cytoprotective from hydrodynamic stress. However, there was no report about the effect of this additive on mass production of RBC in vitro. In this work, the effect of PF on production of red blood cells was determined. Methods; CD34+ cells were isolated from cord blood and cultured without stromal cells for 21-24 days in a medium containing various reagents, chemicals and cytokines. The cell expansion-fold, morphology, and viability were compared between culture from 13 days in the absence or presence of 0.05% PF. Results; The mean expansion-fold of erythroid cells from one CD34+ cell was 41.1 times higher on day 21 compared to that without PF (372,536.1-fold vs 9,069.3-fold, respectively) (N=6, $P<0.01$). In the absence of PF, cell viability was worse as evidenced by low cell number measured by trypan blue stain. The cultured RBCs contained Hb of 12.7 g/dL and MCV 115.9 fL with strong expression of Rh and ABO antigens as well as glycophorin A. The mean HbF was 73.2% on day 17 and decreased to 4.9% on day 24, respectively. Finally, relatively pure RBCs could be harvested by the filtration with little apoptotic fraction in the presence of PF. Discussion; PF was very effective for enhancing viability and recovery of erythroid cells, thus marking an important additive for the mass production of healthy erythroid cells. As PF possesses no growth-stimulating property, it is suggested that the enhanced survival results from the improved availability of PF.

2896/B613

Metabolic Effects of Nicotine Treatment on Human Lung Cells.

K. A. Elwood, L. Rickords, J. M. Labrum; ADVS, Utah State University, Logan, UT

The lung is the first organ to come in contact with nicotine from inhaled smoke (Kitamura 1987), with the principle route of nicotine absorption happening through the alveoli of the lung (Catassi et al. 2008). The genomic and proteomic effects of nicotine on lung cells have been studied extensively for over twenty years. For example, the addition of nicotine to normal murine fibroblast lung cells in culture stimulates the expression of fibronectin, activates protein kinase C and mitogen-activated protein kinases, increases the levels of intracellular cAMP, and causes the phosphorylation and DNA binding of transcription factor CREB. Microarray experiments of bronchial epithelial cells treated with nicotine showed 260 significantly up- and 17 significantly down-regulated genes (Catassi et al. 2008). However, a complete metabolomic description of the effects of nicotine on human lung cells has not been reported previously. Metabolomics offers a unique method for the study of intracellular processes. Since the metabolome, which is the complete complement of metabolites in an organism, is downstream of the genome, transcriptome and proteome, it can provide an amplified and potentially more accurate description of function or phenotype of the organism than the upstream methodologies can. In this study, normal human lung cells (ATCC# CCL-210) were incubated in 0 nM, 20 nM, 200 nM and 2000 nM concentrations of nicotine for 16 hours before trypsinization and collection. Metabolites were then extracted, derivatized and silylated prior to analysis using gas chromatography-mass spectrometry (GC/MS). Results will be presented that show nicotine's effect on key metabolite concentrations between control cells and nicotine treated cells. The identification and comparison of specific small molecule compounds that vary between normal lung cells with and without nicotine treatment will lead to the elucidation of metabolic pathways that are perturbed by the addition of nicotine to the culture media.

2897/B614

Improving Downstream Results with Automated Cell Counting.

K. Remple, D. R. Gray, L. Stone, D. Beacham, B. Mandavilli, R. Batchelor, M. Beaudet; Cellular Analysis Business Unit, Invitrogen Corp, Eugene, OR

Accurate cell enumeration is a critical parameter in a variety of cellular and biochemical applications. Here, we compare results obtained using both automated and manual cell counting methodologies for three assays typical of high throughput screening, high content screening and viral vector transduction experiments. In these applications, maintaining an accurate cell count has proven to result in more consistent and reproducible data. The error associated with traditional methods of manual cell counting is introduced by both the instrument and the user. The error of a conventional hemocytometer counting procedure is as high as 70% while automated cell counting greatly reduces the error by as much as 50% by simply eliminating the variability introduced by the user. A more accurate and consistent cell count improved the downstream results in the following assays. In a homogenous, cell-based assay for potassium ion channel function in human U-2 OS cells, we found that automated cell counting resulted in higher Z' values than numbers obtained by manual counting and decreased assay-to-assay variability. In an image-based microplate assay for phosphor-H2AX in HeLa cells, we found that automated cell counting yielded fewer discounted wells due to fewer samples being statistical outliers. In a viral vector transduction experiment for evaluation of infection efficiency of a baculovirus containing a GFP signaling peptide fused gene into U-2 OS cells, we found the coefficient of variation was minimized. As is evident from these experiments, automated cell counting improves results by providing consistently accurate data that is important for reproducibility and statistical confidence.

2898/B615

Developing a Reliable and Efficient Method for Delivering Nucleic Acids into Hard-to-Transfect Cells in Culture.

A. C. Lauer, B. T. Hughes, B. Gopalakrishnan; Mirus Bio Corporation, Madison, WI

Electroporation is a vital tool used for nucleic acid delivery since it can be used for a wide variety of cell types including hard-to-transfect mammalian primary cells and cell-lines. Research scientists at Mirus Bio are developing an electroporation reagent to facilitate an efficient, easy and reliable method to transfect DNA into hard-to-transfect cell-lines, for which chemical mediated transfection is difficult. This electroporation reagent is a broad spectrum reagent capable of supporting electroporation in multiple cell-lines with high efficiency. Compatibility of the reagent has been demonstrated with most major commercial electroporators. The reagent has been proven capable of facilitating electro-permeabilization of cells and electro-transfer of nucleic acids into hard-to-transfect cells such as immune and neuro-derived cell-lines. This is achieved while causing minimal toxicity and reducing harmful side-effects. The high efficiency electroporation reagent has the potential to facilitate wider applications in the field of in vitro gene-transfer, gene expression studies, and a host of other related areas. Efforts in developing, characterizing and understanding the capabilities of this electroporation reagent, as well as the possible mechanisms underlying the process of electroporation, shall be discussed.

2899/B616

Development of a 3D Culture Model That Mimics the Bone Marrow Subendosteal Niche.

A. P. Barros[1], C. M. Takiya[1], L. Garzoni[2], M. Leal-Ferreira[3], H. S. Dutra[1], M. Meirelles[2], L. B. Chiarini[3], R. Borojevic[1], M. D. Rossi[1]; [1]Histology & Embriology, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil, [2]Ultrastructure and Cell Biology Department, Fiocruz, Rio de Janeiro, Brazil, [3]Biophysics Institute, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

Migration, proliferation and differentiation of hematopoietic stem cells (HSC) are dependent upon a complex three-dimensional bone marrow (BM) microenvironment. In vivo models are complex and immunodeficient mouse chimeras do not reproduce species-specific subtleties, while bidimensional cultures do not represent 3D organization of tissues. In order to establish an in vitro model that mimics the human HSC subendosteal niche, a multicellular spheroid with a central area of osteo-induced BM-derived mesenchymal stromal cells (BM-MSC) and undifferentiated BM-MSC at the periphery were developed. Deposition of extracellular matrix proteins fibronectin, collagen I and IV, laminin, and osteopontin was similar to the in vivo BM microenvironment. Scanty deposition of laminin and collagen IV, a wide distribution of a mesh of fibronectin, and collagen I synthesis exclusively in the central region of osteo-induced BM-MSC were observed. Osteopontin (OP) was found mostly at the interface between the two cell populations. RT-PCR assays indicated that there was a differential expression of CXCL12, WNT5A, and KITLG that was dependent upon the degree of differentiation and culture system. No differences were observed in OP, Angiopoietin-1, DKK1, and Notch receptors JAGGED1 and DELTA1 expression. Migration, lodgment, and proliferation of hematopoietic progenitor cells (HPC) were investigated in co-cultures of magnetically selected cord blood or BM CD34+ cells with multicellular spheroids. CD34+ cells migrated into the spheroids reaching a plateau at 24h that was due to a continuous circulation. They showed a preferential localization at the interface between the two cell population layers. Furthermore, a higher percentage of quiescent CD34+ cells were observed in mixed spheroids compared to CD34+ cells co-cultured with simple undifferentiated BM-MSC spheroids. We propose that this in vitro model mimics the HSC subendosteal niche, and is a useful tool to study mechanisms controlling HSC adherence, transmarrow migration, lodgment and proliferation.

2900/B617

3D Cell Culture for Enhancement of Cell Function.

R. C. Chang[1], W. Sun[1], Q. Liu[2], W. Lau[2]; [1]Mechanical Engineering and Mechanics, Drexel University, Philadelphia, PA, [2]3D Biotek LLC, North Brunswick, NJ

Traditionally, culturing cells on flat polystyrene results in artificial 2D sheets of cells. Cells in vivo, however, experience a 3D environment in which the cell is surrounded by other cells, membranes, fibrous layers, and adhesion proteins. Fundamental cell biology and research is increasingly exploring 3D in vitro cell culture models to uncover patterns of gene expression and other biological phenomena that more closely approximate what is observed in the in vivo microenvironment. In 3D culture, for example, epithelial cells can move and divide more quickly, from integrin dependent links with the ECM, and assume the characteristic in vivo asymmetric shape where the third dimension promotes normal epithelial polarity and differentiation while culturing on 2D induces an artificial polarity between the plate side of the cell and the free face. 3D cultures also show promise as disease models. Herein, a comparison study of biological cell proliferation and function of osteoblasts cultured using two types of 3D InsertTM versus 2D conventional monolayer was conducted. The 3D InsertTM were polymeric 3D porous constructs made of either polycaprolactone (PCL) or polystyrene (PS) fabricated by 3D Biotek, LLC through a freeform fabrication process, with a layered pattern of 0°/90° orientation and defined, controlled 3D porous structures of variable pore sizes ranging from 100 um to 500 um. The experimental data suggests that cell proliferation and functions were higher in 3D InsertTM, where both the PS and PCL cases performed better than 2D monolayer culture owing to over-confluence of cells on the 2D surface where cells can no longer spread, undergo contact inhibition, and leads to loss of viability. The 3D InsertTM shows promise as part of a paradigm shift in cell culture technology to reflect normal cell morphology and behavior for more realistic cell biology and function, in vivo-like morphology, and foster better intercellular interactions.

2901/B618

A New Approach for Drug Discovery and Differentiation Study Using Cutting-Edge 3D Cell Culture System.

A. Lazic[1], F. Ozawa[2], M. Shimomura[3], Y. Miyagawa[4], M. Hiroyama[2,5], N. Kiyokawa[4], A. Umezawa[6], A. Tanoue[5], T. Nakatsura[3], S. Tanaka[2]; [1]InfiniteBio, Inc., Palo Alto, CA, [2]SCIVAX Corporation, Kawasaki-shi, Japan, [3]Hospital East, National Cancer Center, Kashiva, Japan, [4]Department of Development Biology, National Research Institute for Child Health and Development, Setagaya, Japan, [5]Department of Pharmacology, National Research Institute for Child Health and Development, Setagaya, Japan, [6]Department of Reproductive Biology, National Research Institute for Child Health and Development, Setagaya, Japan

Conventional cell-based assays commonly used in the drug discovery process are based on a monolayer (2-dimensional) cell culture system. The obtained drug efficacy data often have a poor correlation with the *In Vivo* data, suggesting a need for a better *in vitro* model to mimic an in vivo environment. NanoCulture Plate (NCP) is a novel experimental system developed for the three-dimensional cell culture. The plate bottom has a special film having a nano-structure pattern which enables spheroid formation of various cell types. At the molecular level, the expression levels of proteins that characterize the in vivo environment were altered in the spheroids compared to monolayer culture. The protein expression of HIF-1, VEGF and AKT was significantly increased in cancer cell spheroids. In addition, 3-D spheroids showed different drug susceptibility depending on the combination of specific drug and cell types compared to monolayer cells. Furthermore, we established a method for 3D primary culture of pancreatic cancer and hepatocellular carcinoma (HCC) from clinical samples obtained from patients at National Cancer Center Hospital East, Japan. The formation of spheroid on the NCP was confirmed in approximately 90% of the samples and the excessive growth of fibroblasts was not observed. In addition, expression of HCC marker Glypican-3 (GPC3) was observed in spherical structure of the cancer cells. NCP is also useful for spheroid formation of normal cells such as hepatocytes, adipocytes, and mesenchymal stem cells. On NCP, the characteristics of cellular differentiation in spheroid were maintained and emphasized compared to monolayer cultured cells. Hepatocytes secreted higher levels of albumin, a differentiation marker, and showed upregulation of the cytochrome P450 gene expression. Stem cells form 3D spheroids on the NCP, differentiate to adipocytes, after the induction, with a higher content of triglyceride, and highly express differentiation marker-genes. These results suggest that spheroids on NCP have *In Vivo* like characteristics. This *in vitro* 3D culture system offers a new approach for more relevant cell based assays in drug screening to obtain *In Vivo* comparable results.

2902/B619

Sciatic Nerve Conditioned Medium Favors Glia Over Neurons in Hippocampal Cell Cultures.

L. Garcia, M. Longart, C. Castillo; Instituto de Estudios Avanzados, Sartenejas, Venezuela

After injury, the Peripheral Nervous System (PNS) has a better regeneration process than the Central Nervous System (CNS). Part of this difference is due to the kind of glial cells present and the factors liberated by these cells in each system. Oligodendrocytes and astrocytes are the main glial cells in the CNS, whereas Schwann cells are the main glial cells in the PNS. In this work, we evaluated the effect of a rat-sciatic nerve conditioned medium during Wallerian degeneration (CM) on primary hippocampal cultures (CNS). Primary hippocampal cultures in CM were immunostained with an antibody against glial fibrillary acidic protein (GFAP) and Microtubule Associated Protein 2 (Map2) to evaluate the astrocyte and neuronal populations in cultures at different times. We found that CM increased the proportion of astrocyte in the culture (from 11.37% in control cultures to 63.01% in CM cultures), this increase was subjected to the age of the culture. Additionally, CM promoted the appearance of type-1 astrocytes, absent in the control condition. Concomitantly, this increased was accompanied by a decrease of the neuronal population. These results suggest that sciatic nerve releases factors that favor glial development versus neurons

2903/B620

In Vitro Culture of Primary Equine Hoof Keratinocytes and Changes in Cell Proliferation and Apoptosis in Response to Strain.

A. M. Chaudhry, J. J. Thomason, J. Petrik; Biomedical Sciences, Ontario Veterinary College, University of Guelph, Guelph, ON, Canada

The horse's hoof shares an important property with the skeleton: it is a mechanical structure that has the ability to respond to changes in the way it is loaded. One type of response is for the external shape of the hoof capsule to alter, thereby changing the mechanical behavior of the whole hoof in response to externally applied loads. The objective of this study was to extract cells from the coronary band of the hoof and maintain primary cultures of equine hoof keratinocytes (EHK). These cultures were then evaluated to determine relationships between gross growth rates and local loading in vitro. Immunocytological stains for keratin and morphological examination confirmed a new method of extracting and maintaining cultures of fibroblast free EHK cells. EHK cells were successfully extracted and maintained from 4 horses and have survived 30 passages in vitro. Cells were then subjected to hydrostatic pressure directly corresponding to ongoing in vivo research and evaluated for alterations in protein expression, apoptosis, and proliferation. In vivo experiments included assessments of loading in the hoof which influenced the mechanical structure of the hoof wall. This loading plays a significant role in the pathology of hoof disease and overall performance of the horse. Results of these experiments revealed that varying loads of hydrostatic pressure influence cell death, growth and protein expression in vitro. Knowledge of in vitro loading associated with equine hoof wall furthers the understanding of mechanisms to aid in the prevention and treatment of equine hoof maladies.

Host-Pathogen Interactions (2904 – 2924)

2904/B622

About the Role of P38 MAPK and Endocytosis in Promoting Recovery of Pore-forming Toxin-treated Cells.

M. Bischofberger, M. Gonzalez, B. Frêche, F. van der Goot; Life Sciences, EPFL, Lausanne, Switzerland

Pore-forming toxins (PFTs) are secreted proteins that contribute to the virulence of a great variety of bacterial pathogens by perturbing the plasma membrane integrity of the target cell. Work by our lab and others have highlighted that mammalian cells defend themselves from pore-formation by activating specific pathways, most importantly the p38 MAP kinase and SREBP, the central regulator of lipid biosynthesis. These pathways have been shown to promote cell survival. However, it is not clear how cells activate these pathways and how they contribute to cell survival. Recently, p38 has been involved in modulating endocytosis. We therefore investigate if endocytosis could be a defense mechanism activated by p38 to remove toxins from the surface. We show that the PFT-induced decrease in cellular potassium is necessary and sufficient to trigger the phosphorylation of p38. Interestingly, we also find a concomitant arrest in protein translation, due to the phosphorylation of eukaryotic Initiation Factor 2α (eIF2α). Moreover, we observe for the first time the recovery of intracellular potassium levels after transient treatment with aerolysin, a pore-forming toxin, and the concomitant recovery of protein synthesis in colon cancer cells. Intriguingly, recovery of intracellular potassium indeed is p38-dependent. Finally, we show that endocytosis of the toxin is indeed occurring

after toxin-treatment and is p38-dependent. Based on these findings we suggest that the p38 MAP kinase is activated after pore formation in a potassium-dependent manner, as well as translational arrest through eIF2α. P38 is then responsible for the recovery of the plasma membrane integrity through an enhanced endocytosis of the toxins on the surface, leading to the prolonged cell survival observed in earlier publications.

2905/B623

Enterocyte Microvilli Generate Vesicles That Interact with Luminal Microbes and Detoxify Bacterial Lipopolysaccharide.

R. McConnell[1], J. N. Higginbotham[2], R. Coffey[1,2], D. A. Shifrin[1], D. L. Tabb[3], M. Tyska[1]; [1]Cell & Developmental Biology, Vanderbilt University Medical Center, Nashville, TN, [2]Medicine, Vanderbilt University, Nashville, TN, [3]Biomedical Informatics, Vanderbilt University, Nashville, TN

The enterocyte brush border is composed of a hexagonally packed array of microvilli that has classically been viewed as a passive cytoskeletal scaffold, which serves to increase apical membrane surface area of the intestinal epithelium. However, recent work with isolated brush borders indicates that exposure to ATP stimulates a myosin-1a powered release of apical membrane vesicles from the tips of microvilli, a process we now refer to as brush border membrane shedding. Our goal with the present study was to investigate the functional role of vesicles produced during this process in vivo. Using small intestinal luminal contents as starting material, we employed a combination of biochemical fractionation and fluorescence activated vesicle sorting to isolate a population of vesicles that are enriched in a number of catalytically active enzymes ordinarily associated with the microvillar membrane. One of the most highly enriched components in this vesicle fraction is intestinal alkaline phosphatase, a brush border enzyme recently implicated in mucosal defense against toxic bacterial products. Consistent with a role in host defense, microvillus-derived vesicles physically interact with luminal bacteria, dephosphorylate purified bacterial lipopolysaccharide, and protect cultured epithelial cells from the pro-inflammatory signaling normally stimulated by this microbial product. Intriguingly, vesicle production is stimulated by feeding, and therefore may contribute to the beneficial effects of enteral nutrition in maintaining gut mucosal barrier function. Mice lacking myosin-1a exhibit dramatic defects in both vesicle size and composition, suggesting that brush border membrane shedding is misregulated in the absence of myosin-1a. Thus, we propose that myosin-1a powers brush border membrane shedding in vivo, and that this activity represents a novel, previously uncharacterized mechanism for distributing host defense machinery throughout the intestinal lumen.

2906/B624

The Role of Ferroportin in Macrophage-Mediated Immunity.

E. E. Johnson[1], A. Sandgren[2], M. Murray[2], M. Wessling-Resnick[1]; [1]Genetics and Complex Diseases, Harvard School of Public Health, Boston, MA, [2]Epidemiology, Harvard School of Public Health, Boston, MA

Ferroportin (FPN) plays a key role in iron homeostasis through modulation of iron egress from duodenal enterocytes and macrophages. Infection and inflammation down-regulate FPN to limit iron efflux from macrophages thus sequestering this essential nutrient from invading microbes. It is unknown whether FPN contributes to other macrophage-mediated immune responses. In particular, the association of iron metabolism and nitric oxide (NO) production by macrophage inducible nitric oxide synthase (iNOS) remains the subject of debate. Induction of NO synthesis by iNOS is a key inflammatory response mechanism triggered by cytokines and various microbial insults including lipopolysaccharide (LPS). J774 murine macrophages over-expressing FPN were used to investigate the influence of iron efflux on NO production in response to challenge with virulent Mycobacterium tuberculosis (M.tb) and LPS. Over-expression of FPN significantly impaired intracellular M.tb growth. When challenged with LPS or M.tb, control macrophages increased NO synthesis as expected. However, macrophages over-expressing FPN had significantly impaired NO production in response to LPS or M.tb. Western blot and qRT-PCR analysis showed that increased NO synthesis in control cells was accompanied by increased iNOS mRNA and protein expression. Surprisingly, up-regulation of iNOS protein was markedly reduced when J744 cells over-expressing FPN were challenged with LPS or M.tb. These results suggest that iron efflux by FPN limits not only intracellular bacterial growth but also macrophage-mediated immune responses.

2907/B625

Effect of Recombinant Lectin-Like Domain of Thrombomodulin on the Lipopolysaccharide-Induced Signaling Pathways.

G. Shi[1,2], C. Shi[1,2], H. Wu[1,2]; [1]Department of Biochemistry and Molecular Biology, National Cheng Kung University, Tainan, Taiwan, [2]Cardiovascular Research Center, National Cheng Kung University, Tainan, Taiwan

Previous report has demonstrated that thrombomodulin (TM) appears to exhibit anti-inflammatory ability through both protein C dependent and independent pathways. This study aims at investigating the effect of recombinant lectin-like domain of TM (rTMD1) on the lipopolysaccharide (LPS)-induced signaling pathways. rTMD1 was prepared and purified using both Pichia pastoris and mammalian protein expression systems. Effects of rTMD1 treatment on the LPS-induced inflammatory mediator production and signaling pathways were studied. rTMD1 but not recombinant TM domain 2 (TMD2) containing epidermal growth factor-like domain 1, 2, 3 (rTMD2/EGF123) dose-dependently inhibited tumor necrosis factor-α and nitric oxide productions in RAW 264.7 cells stimulated with LPS. Phosphorylation and degradation of IkBα occurred in RAW 264.7 cells stimulated with LPS. The effect was totally reversed by rTMD1. The LPS-induced nuclear translocation of nuclear factor-kB was also inhibited in a dose-dependent fashion by rTMD1. LPS-induced phosphorylation of extra-cellular signal-regulated kinase 1/2 and p38 was also inhibited by rTMD1. In addition, inducible nitric oxide synthase induction in the RAW 264.7 cells by LPS was inhibited by rTMD1. In conclusion, rTMD1 inhibited LPS-induced inflammatory mediator production and modulated LPS-induced mitogen-activated protein kinase and nuclear factor-kB signaling pathway activations and inducible nitric oxide synthase expression in macrophages.

2908/B626

Using Infection to Analyze Septin Function.

S. Mostowy[1], T. Tham[1], S. Janel[2], F. Lafont[2], J. Pizarro-Cerdá[1], P. Cossart[1]; [1]Unité des Interactions Bactéries-Cellules, Institut Pasteur, Paris, France, [2]Lille Biology Institute, Lille Pasteur Institute, Lille, France

Septins are a family of conserved GTPases that form filaments and are required in many organisms for a variety of processes including cytokinesis. We previously identified SEPT9 as associated with Listeria monocytogenes at early stages of infection in mammalian cells. Given the well-established association of septins with the cytoskeleton, and the importance of the cytoskeleton in bacterial invasion, we are investigating septin function during infection of invasive bacteria in non-phagocytic cells. Analyses employing multiple pathogens and cell biology tools has revealed previously uncharacterized roles for septins in basic cell architecture, particle entry, and actin-based motility. Current research aims are focused upon analysis of septin function during precise stages of host-pathogen interaction. A complete characterization of septin biology during infection could provide new targets to fight against intracellular pathogens, aid in initiating investigations of similar mechanisms in other disease states, and lead to new concepts in cell biology.

2909/B627

Blockade of 4-1BB/4-1BB Ligand Interactions Increases Survival in Polymicrobial Sepsis Induced by Cecal ligation and Puncture.

T. Q. Nguyen, M. S. Park, S. B. Kim; Department of Pathology, Ulsan University Hospital, School of Medicine, University of Ulsan, Ulsan, South Korea

Sepsis, a leading cause of death worldwide, involves pro-inflammatory responses and inefficient bacterial clearance. 4-1BB (CD137), a member of the TNFR superfamily, is a T-cell-costimulatory receptor expressed on activated T cells, dendritic cells, neutrophils and NK cells. 4-1BB binds 4-1BB ligand (4-1BBL), a high affinity ligand presented on a variety of antigen-presenting cells. In a cecal ligation and puncture (CLP) model of septic peritonitis, we found that 4-1BB-deficient (4-1BB-/-) mice were significantly protected against CLP-induced mortality compared with control 4-1BB+/+ mice. 4-1BB-/- mice exhibited lower IL-10 and chemokine/pro-inflammatory cytokine (MCP-1, IL-6, TNF-α, IL-12) levels, higher bacterial clearance, and greater survival after CLP compared with control 4-1BB+/+ mice. Protection of 4-1BB-/- mice after CLP was associated with a greater number of peritoneal neutrophils and macrophages than in control 4-1BB+/+ mice. Remarkably, administration of anti-4-1BBL monoclonal antibody into 4-1BB+/+ mice that blocked 4-1BB/4-1BBL interactions increased bacterial clearance, survival rate, neutrophil and macrophage recruitment into the peritoneum, and decreased IL-10 and chemokine/pro-inflammatory cytokine levels after CLP. These data demonstrated that the absence of 4-1BB protected mice from septic peritonitis and blocking 4-1BB/4-1BBL interactions in wild-type mice resulted in the responses similar to those in 4-1BB-/- mice during polymicrobial sepsis.

2910/B628

Curcumin Inhibits Peptidoglycan-Mediated Inflammatory Response in Human Cystic Fibrosis Bronchial Epithelial Cells through Inhibition of TLR2 Gene Expression.

Y. Ohira, T. Shuto, S. Shimasaki, K. Watanabe, M. Suico, H. Kai; Molecular Medicine, Graduate School of Pharmaceutical Sciences, Kumamoto University, Kumamoto, Japan

Cystic fibrosis (CF), the most common lethal inherited disorder caused by mutation in the gene encoding the CF transmembrane regulator (CFTR), is characterized by chronic inflammation that ultimately lead to death from respiratory failure. In CF patients, up-regulation of toll-like receptor-2 (TLR2), a pattern recognition receptor that senses CF-pathogenic bacteria *S. aureus* peptidoglycan (PGN), in airway epithelial cells is observed, and enhances proinflammatory responses towards PGN that results in detrimental effects in CF patients. Therefore, the agents that control TLR2 expression and their signaling could be the ideal inflammatory modulator in CF. In the present study, we investigated the effects of curcumin, a well known anti-inflammatory agent derived from the curry spice turmeric, on the TLR2 signaling and their expression in CF bronchial epithelial cell line, CFBE41o- cells. We first examined the effect of curcumin on the responsiveness against various TLR ligands in CFBE41o- cells. Our quantitative RT-PCR analysis data showed that bacterial-derived peptidoglycan (PGN) and gram-negative bacterium lipopolysaccharide (LPS), specific ligand for TLR2 and TLR4 respectively, strongly induce IL-8 mRNA in CFBE41o- cells and only PGN-induced TLR2-dependent IL-8 induction is markedly inhibited by curcumin treatment (40μM). In accordance with this reduced responsiveness against PGN, reduced expression of surface TLR2 protein in curcumin-treated CFBE41o- cells was observed. Moreover, treatment of CFBE41o- cells with curcumin also suppressed TLR2 mRNA expression in time-dependent manner. Thus, our data demonstrate the possible application of curcumin treatment on regulating TLR2-derived hyperinflammatory responses in CF bronchial epithelial cells.

2912/B630

In Vitro Characterization of Moraxella Catarrhalis Lgt3.

I. A. Faglin, J. Tiralongo, J. Wilson, I. Peak; Institute for Glycomics, Griffith University, Gold Coast, QLD, Australia

Moraxella catarrhalis is currently the third leading cause of otitis media in children and induces lower respiratory tract infections in adults with chronic pulmonary disease. A major outer membrane component of this pathogen, the lipooligosaccharide (LOS) molecule has been suggested as an important virulence factor in the pathogenesis of M. catarrhalis infections. This study reports the overexpression and in vitro characterization of Lgt3, a beta glucosyltransferase involved in the assembly of the oligosaccharide portion of M.catarrhalis LOS. The lgt3 gene was amplified by PCR and cloned into the expression vector pET19b. Functional Lgt3 was successfully expressed and purified, and its biochemical properties were investigated in-vitro using radioactive assays. Lgt3 activity was assessed by autoradiography and liquid scintillation counting. Lgt3 was only able to mediate the addition of glucose onto a specific LOS structure, indicating high acceptor specificity. Although UDP-Glc was the preferred donor substrate, the recombinant enzyme showed donor promiscuity toward UDP-Gal. Optimal pH and temperature were 6.8 and 37 degrees Celsius respectively and the activity was maximal in buffer containing magnesium. The enzyme activity was slightly reduced when manganese was used instead and halved when calcium or no ion was used.

2913/B631

The B-Subunits of Shiga-Like Toxins 1 and 2 Promote VWF Secretion via a Cholesterol Dependent Mechanism in Human Endothelial Cells.

J. Huang, E. Sadler; Washington University School of Medicine, St. Louis, MO

Hemolytic-uremic syndrome (HUS) is a disease characterized by microangiopathic hemolytic anemia, thrombocytopenia and renal failure. In diarrhea-positive HUS (D+HUS), renal endothelial damage is largely mediated by the infectious agent Shiga toxin (Stx). Stx and its related toxins (Stx1 and Stx2) are composed of a single A subunit, which inactivates the 28S ribosomal RNA, and pentameric B subunits (B5), which bind to cell surface globotriaosylceramide (Gb3) receptors. Previous studies have shown that Stx1 and Stx2 holotoxins cause human endothelial cells to secrete von Willebrand factor (VWF) multimers, and induce VWF-dependent microvascular thrombosis in ADAMTS13 null mice. Here we investigated the effect of catalytically inactive B5 subunits of Stx1 and Stx2 on human endothelial cells, focusing on the acute responses. Unexpectedly, we found that at nanomolar concentrations, the B5 subunits of Stx1 and Stx2 specifically and dose dependently stimulated the release of VWF from human endothelial cells. These VWF multimers formed extended "strings" under laminar flow on endothelial surface five minutes following addition of these B5 subunits. Anti-Gb3 IgM and cholera toxin B5 could also induce VWF string formation, but a synthetic Gb3 analog (DAISY compound) inhibited this effect of Stx B5, suggesting that binding and clustering of Gb3 receptors within lipid rafts are required. Cholesterol depletion by methyl beta cyclodextrin inhibited the induction of VWF string formation by Stx B5 but not by histamine. Since Stx can enter cells via multiple pathways, we tested whether this effect of Stx B5 depends on a specific endocytosis route. We found that inhibition of clathrin dependent endocytosis had no effect, whereas sequestration of cholesterol by filipin almost complete abolished Stx B5-induced VWF secretion. Because filipin also disrupts endothelial caveolae, the effect of filipin suggests that Stx B5 may interact with caveolae. These data show that the B5 subunits of Shiga-like toxins can acutely activate endothelial signaling pathways by a novel mechanism that is independent of the delayed ribotoxic effects of the Stx A subunit. Such knowledge may be important for a better understanding of HUS pathogenesis.

2914/B632

The Catalytic Subunit of Shiga-Like Toxin 1 Interacts with Ribosomal Stalk Proteins P0, P1, and P2.

A. J. McCluskey[1,2], G. Poon[2], J. Gariepy[1,2]; [1]Pharmaceutical Sciences, University of Toronto, Toronto, ON, Canada, [2]Cancer Genomics and Proteomics, Ontario Cancer Institute, Toronto, ON, Canada

Shiga-like toxin 1 (SLT-1) is a type II ribosome-inactivating protein where its A1 domain blocks protein synthesis in eukaryotic cells by catalyzing the depurination of a single adenine base in 28S rRNA. The molecular mechanism leading to this site-specific depurination event is thought to involve interactions with eukaryotic ribosomal proteins. Here we present evidence that the A1 chain of SLT-1 binds to the ribosomal proteins P0, P1, and P2. These proteins were identified from a HeLa cell lysate by tandem mass spectrometry, and subsequently confirmed to bind to SLT-1 A1 chain by yeast-2-hybrid and pull-down experiments using candidate full length proteins. Moreover, the removal of the last 17 amino acids of either proteins P1 and P2 abolishes their interaction with the A1 chain whereas P0 lacking this common C-terminus still binds to the A1 domain. In vitro pull-down experiments using fusion protein-tagged C-terminal peptides corresponding to the common 7, 11, and 17 terminal residues of P1 and P2 confirmed that the A1 chain of SLT-1 as well as the A chain of ricin bind to this common C-terminal peptide motif. These results suggest a role for the ribosomal stalk in aiding the A1 chain of SLT-1 and other type II ribosome-inactivating proteins such as ricin in localizing their catalytic domain near the site of depurination in the 28S rRNA.

2915/B633

Myosin X Facilitates Shigella Filopodia Formation and Cell-to-Cell Spread.

E. Bishai[1], G. Sidhu[1], A. Bohil[2], R. Cheney[2], F. Southwick[1]; [1]Medicine, Infectious Diseases, Univ of Florida, Gainesville, FL, [2]Univ of North Carolina, Chapel Hill, NC

The intracellular pathogen Shigella flexneri forms filopodia in order to spread from cell to cell. We have found that as Shigella form filopodia, myosin-X (Myo10) concentrates along the sides of the bacteria. Listeria monocytogenes, another intracellular pathogen that induces filopodia formation in host cells does not attract Myo10. To assess the functional significance of this finding scanning electron micrographs of Shigella-infected HeLa cells were subjected to RNAi knockdown of Myo10 and compared to cells treated with control RNAi. Knockdown of Myo10 was associated with the formation of broad-based filopodia, as compared to control cells in which filopodia were attached by a thin stalks. Myo10 knockdown also significantly reduced the length of Shigella-induced filopodia (mean length 8.6 ± 0.5 um vs. control 11.1 ± 0.5 um), and resulting in a 26% decrease in plaque formation. Live video of GFP-full-length Myo10 and a head-coil-coil GFP-construct revealed that both proteins cycle backward and forward along the sides of the bacteria within filopodia. Transfection of Cos7 (contain low levels of Myo10) with full-length Myo10, but not the head construct, increased filopodia length (mean length 14.6 ± 0.5 um vs. 11.6 ± 0.4 um). Enhanced filopodia formation required the PH domain of the tail, but not the FERM domain. GFP-Myo10-PH localized to the cell membranes including filopodia, while GFP-Myo10-MyTh4-FERM did not. These findings suggest Myo10 generates the force to enhance filopodia formation by binding its head region to Shigella-associated actin filaments, and its PH tail domain to the host cell membrane. This same mechanism is likely to contribute to Myo10-induced filopodia formation in uninfected cells.

2916/B634

Interaction of *Candida Albicans* pH-Regulated Antigen 1 (Pra1p) with Leukocyte Integrin αMβ2 Modulates the Host Response to Infection.

C. Drumm[1], W. A. Fonzi[2], D. A. Soloviev[1]; [1]Molecular Cardiology, Cleveland Clinic, Cleveland, OH, [2]Department of Microbiology and Immunology, Georgetown University, School of Medicine, Washington, DC

Candida albicans is a common opportunistic fungal pathogen and is the leading cause of invasive fungal disease in immunocompromised individuals. The induction of cell-mediated immunity to *C. albicans* is of critical importance in host defense and the prime task of cells of the innate immune system. From *in vitro* studies, we identified integrin αMβ2 (Mac-1, CD11b/CD18) as the principle leukocyte receptor for recognition, recruitment, adhesion and killing/phagocytosis of *C. albicans*, and the major ligand for αMβ2 on *C. albicans* as the pH-regulated antigen 1 (Pra1p, Fbp1). Employing normal (WT) and Pra1p-deleted strains of *C. albicans* and polymorphonuclear neutrophils (PMNs) isolated from WT and αMβ2 deficient mice, we found that WT PMNs were successful only in killing/phagocytosis of WT *C. albicans* (expressing Pra1p). If either Pra1p or αMβ2 were deleted, *C. albicans* killing was markedly suppressed. In an *In Vivo* murine model of *C. albicans* peritonitis, elimination of Pra1p caused a 4-fold increase of fungal viability in the peritoneum during the first 6 hrs of infection,

from 16% ± 10% to 65% ± 12% ($P < 0.05$). Restoration of the PRA1 gene into *C.albicans* decreased fungal viability to the level observed with WT *C. albicans*. In contrast, mice lacking αMβ2 failed to significantly reduce WT *C. albicans* levels during the first 6 hrs of infection (67% ± 12% viable fungal cells). Indeed, the αMβ2 deficient mice failed to significantly reduce the infection of all *C. albicans* strains tested, either with or without Pra1p expression (fungal viability was in the range of 65% to 75%). Additionally, removal of either αMβ2 or Pra1p reduced leukocyte recruitment into peritoneum during the first 6 hrs of infection by 2-3 fold. Our results indicate the importance of Pra1p - integrin αMβ2 interaction in the initial response of host innate immune system to *C. albicans* infection.

2917/B635

Activation of Rho GTPase Signaling Pathways in Human Oral Keratinocytes by Pathogenic *C. Albicans.*

T. Woellert[1,2], C. Rollenhagen[3], P. Sundstrom[3], G. Langford[1,2]; [1]Department of Biology, Syracuse University, Syracuse, NY, [2]Biology Department, University of Massachusetts, Amherst, MA, [3]Department of Microbiology, Dartmouth College, Hanover, NH

To determine the specific changes in the Rho signaling pathway of human oral keratinocytes (OKF6/TERT-2) when exposed to *Candida albicans* virulence factors, we co-cultured OKF6/TERT-2 cells with pathogenic *C. albicans*. These virulence factors caused rapid changes in F-actin dynamics in OKF6/TERT-2 cells within 3-6 hours and stimulated cell migration. Actin remodeling resulted from the sequential activation of Rho GTPase family members. In untreated keratinocytes, Cdc42 was active as indicated by the presence of filopodia. Treatment of keratinocytes with wild-type *C. albicans* activated RhoA as indicated by the formation of actin stress fibers and focal adhesions at 3 hours. Rac1 was activated at 6 hours and lamellipodia appeared. Inhibition of Rho/ROCK or Rac1 pathway prevented actin remodeling caused by *C. albicans*. As a control, actin remodeling was not observed in the presence of the non-pathogenic yeast *Saccharomyces cerevisiae*. *C. albicans* mutant *SAP4-6Δ* failed to activate the Rho GTPase signaling pathway indicating that the Rho pathway was triggered only when keratinocytes were treated with aspartyl proteinase secreting hyphae-forming *C. albicans*. The induction of actin remodeling in keratinocytes by pathogenic *C. albicans* may constitute an important part of the mechanism for invasion of the oral epithelium in candidiasis.

2918/B636

***Toxoplasma Gondii* Regulation of the Host Cell Transcription Factor, Hypoxia Inducible Factor 1.**

M. Wiley, I. Blader; University of Oklahoma Health Sciences Center, Oklahoma City, OK

Toxoplasma gondii is an obligate intracellular parasite that can cause devastating disease in fetuses and immune compromised patients. New drugs to treat *Toxoplasma* infections are needed because current therapies to these infections have severe side effects and cannot eliminate the parasite. To identify new drug targets, our laboratory focuses on identifying host cell processes critical for parasite growth. We previously reported that the hypoxia inducible factor 1-α (HIF1) subunit of the HIF1 host cell transcription factor is upregulated by infection and is necessary for *Toxoplasma* growth. Under basal conditions, this subunit is constitutively expressed and then rapidly targeted for proteasomal degradation after two proline residues are hydroxylated. This hydroxylation is catalyzed by a family of proteins named prolyl hydroxylase domain containing enzymes 1-3 (PHDs). The overall goal of this study was to define how *Toxoplasma* regulates HIF1α protein levels. Here, we show that *Toxoplasma* does not affect HIF1α transcription or translation, but rather increases its stabilization. To define, how *Toxoplasma* increases HIF1α protein stability, we tested whether infection blocked HIF1α hydroxylation. First, we transfected cells with a luciferase reporter in which luciferase is fused to the domain of HIF1α containing the proline residues that are substrates for the PHDs. We found that infection led to an increase in luciferase reporter expression. In addition, Western blotting with antibodies that only recognize prolyl hydroxylated HIF1α showed that HIF1α is not prolyl hydroxylated in parasite-infected cells. Finally, we demonstrate that *Toxoplasma* specifically down regulates expression of PHD2, which has been defined by others as the primary PHD that regulates HIF1α. Together, these data indicate that *Toxoplasma* regulates a host cell transcription factor critical for its growth by down regulating expression of its negative regulator.

2919/B637

The Involvement of Oxidative Burst in Cell Death, Pathogen Propagation and Scopoletin Production in Hevea Brasiliensis-Phytophthora Palmivora Interaction.

N. Chirapongsatonkul, N. Churngchow; Biochemistry, Prince of Songkla University, Hatyai, Songkhla, Thailand

The involvement of oxidative burst, the generation of superoxide anion (O2-) and hydrogen peroxide (H2O2), in other defense-related responses was verified in Hevea brasiliensis-Phytophthora palmivora interaction by using the oxidative burst inhibitors. The inhibitors of NADPH oxidase (Diphenylene iodonium, DPI) and Cu/Zn superoxide dismutase (SOD) (Diethyldithiocarbamate, DDC) were pre-treated on H. brasiliensis (BPM-24 cultivar) leaves 24 hours prior to being inoculated with zoospores of oomycete P. palmivora (5x106 zoospores/ml). The generation of O2-, H2O2 and cell death was measured by nitroblue tetrazolium (NBT), 3, 3'-diaminobenzidine (DAB) and trypan blue staining, respectively. The pathogen propagation including the density of mycelia and sporangia was observed microscopically whereas scopoletin (Scp, phytoalexin produced in H. brasiliensis) was determined spectrofluorometrically. Pretreatment of the leaves with DPI abolished the blue formazan precipitate representing O2- generation whereas DDC enhanced O2- accumulation. Pretreated with either DPI or DDC resulted in a significant reduction of the brown precipitate representing H2O2 accumulation. Both DPI and DDC also caused the reduction of hypersensitive response (HR) cell death followed by a significant increase of lesion size, spreading of the pathogen, mycelium density and amount of sporangia compared to zoospore treatment (without inhibitors). However, Scp accumulation showed a clear decline after DPI pretreatment (65% reduced) and a smaller reduction (20% reduced) after DDC pretreatment. Therefore, these results suggest that in our model, O2- was generated via NADPH oxidase whereas H2O2 was partly synthesized by dismutation from O2- via the action of SOD and from other pathways. Both O2- and H2O2 were involved in the control of HR cell death and in preventing pathogen spread whereas O2- rather than H2O2 was necessary for the production of Scp.

2920/B638

Recognition and Adhesion Process to Neuronal, Lung and Bone Cells by Fungal Surface Manoproteins.

M. Sabanero Lopez[1], G. Sandoval Bernal[1], K. Barbosa Sabanero[1], G. Barbosa Sabanero[2], M. Shibayama Salas[3], V. Tsutsumi Fujiyoshi[3];

[1]Instituto de Investigacion en Biologia Experimental, Universidad de Guanajuato, Guanajuato, Mexico, [2]Instituto de Investigaciones Medicas, Universidad de Guanajuato, Léon, Mexico, [3]Infectómica y Patogénesis Molecular, Centro de Investigación y de Estudios Avanzados del IPN, México DF, Mexico

Sporothrix schenckii is a pathogenic fungus, etiological agent sporotrichosis, mycosis that evolve to systemic complication in immunocompromised patient. S. schenckii may infect SNC, pulmonary and osteoarticular tissues, unknown the mechanism involved tissue tropism. The adherence of pathogenic microorganism to host represents a critical step in the process of infection. In order to determinate the mechanisms of adhesion of S. schenckii to tissues, in this work, we analyzing the fungal adhesion process. Using different cells in culture: neuronal (MNS-SK), bone (MG-63) Lung (A549), epithelium (L929) and endothelial (HeK293), we analyze 1) Adhesion kinetic of yeast to cells. In 1 h of interaction of fungus with cells, S. schenckii present major adhesion capability to neuronal, pulmonary and epithelial cells (91.2 % ± 1.1, 88.3 % ± 2.1 and 85 % ± 0.7 respectively). 2) Using surface cell wall proteins labeling with 1 mM sulfo-NHS-LC biotin and later extraction process with boiling in 2% SDS, this revealed 14 bands of surface proteins (Mr > from 30 to 220 kDa), 3) Using ConA-HRP stain in blot, we determine the mannoproteins nature of molecules from cells walls, 4) To identify the surface glycoproteins of yeast cells of S. schenckii (SgSs) implicated in adhesion to cells, we use ligand-like assays, To this, SgSs previous labeled with biotin, were placed in interaction with the cells.The fungal biotinilated proteins that interacted with cells, were identified in blot using Streptavidin - HRP reaction. The results showed, SgSs involved in the recognition of the cells are: a) Mr> 180, 115, 100, 90 kDa., neuronal, b) Mr> 115, 100, 90 kDa., lung, c) Mr>220, 180, 115, 100, 90 kDa., epithelial and d) Mr>115 kDa., endothelial and bone. Finally, 5) SgSs that participate in the adhesion process, were used to produce polyclonal antibodies that had the capability to inhibit the yeast adhesion to neuronal, pulmonary and epithelial cells (25 % ± 3.2, 35 % ± 2.9 and 54 % ± 1.9 respectively). We propose these surface molecules could be receptors like-adhesins and mediate in the adhesion to host cell. This work was supported by grant from DINPO - UG No. - 18 to MSL.

2921/B639

Shape for Function: the Apicomplexan Parasite Theileria Annulata Controls Cytoskeleton Dynamics of Its Host Cell.

M. Baumgartner, D. Dobbelaere; Clin. Res. Vet. Publ. Health, University of Bern, Vetsuisse Faculty, Bern, Switzerland

The eukaryotic cytoskeleton coordinates a broad range of cellular processes including cell motility. The versatility of the cytoskeleton is prone to selective exploitation by intracellular pathogens and the regulation of host cell motility is targeted by apicomplexan parasites for their dissemination. We are interested in how apicomplexan parasites interact with the host cell actin cytoskeleton and how this interaction leads to altered host cell motility that favors parasite propagation. Theileria species are apicomplexan parasites closely related to Plasmodium and Toxoplasma species parasites, and cause a lymphoproliferative disorder in cattle. T. annulata infects B cells and macrophages, induces their de-differentiation and immortalisation, and promotes an invasive phenotype in the host cell that is similar to that of tumor cells. Unlike irreversible oncogenic alterations, however, transformation induced by Theileria is reversible and allows the identification of de-differentiation and transformation mechanisms. We are focusing on the regulation of host cell kinases by the parasite and their role in actin cytoskeleton dynamics in general and in the assembly and turnover of macrophage podosomes in particular. By using green-fluorescent protein-labeled actin in live cell imaging, we found that the parasite promotes podosome assembly in response to cell-matrix contact and changes motile properties of infected cells. These changes are accompanied by decreased accumulation in podosomes of cortactin and Arp2, a component of the actin polymerization factor Arp2/3. Degradation and altered phagocytosis of fluorescently labeled matrices indicates that the parasite targets host cell components that coordinate matrix degradation and phagocytosis. By phospho-antibody array analysis, we identified a number of phosphoproteins that are specifically regulated in a parasite-dependent manner, including actin regulators, adhesion proteins and kinases. These findings show that protozoan parasites selectively modulate the function of host cell signaling pathways and structural proteins that coordinate cytoskeleton dynamics, cell migration and endocytosis.

2922/B640

Chlamydia Trachomatis Infection Induces Spindle Pole Disorganization in Cells Expressing HPV Oncoproteins.

A. Knowlton, S. Grieshaber; Oral Biology, University of Florida, Gainesville, FL

Chlamydiae are gram negative, obligate intracellular bacterial organisms, and Chlamydia trachomatis is the etiologic agent of the most commonly reported sexually transmitted disease in the United States. Chlamydial infections have also been epidemiologically linked to cervical cancer in patients simultaneously infected by human papillomavirus (HPV). Chlamydiae undergo a biphasic life cycle that takes place inside a parasitophorous vacuole termed an inclusion. The inclusion associates very closely with host cell centrosomes, and this association is dependent upon dynein. We have previously reported this interaction induces supernumerary centrosomes in infected cells, leading to multipolar spindles and inhibiting accurate chromosome segregation during mitosis. Centrosome amplification is characteristic of many human cancers. Through Chlamydia's dominant interaction with dynein and host cell centrosomes, we hypothesize that not only is numeral centrosome integrity affected, but also the cell's ability to cluster supernumerary centrosomes. High risk HPV type 16 oncoproteins E6 and E7 contribute to cervical cancer by cooperating to induce abnormal centrosome numbers; however pre-cancerous cells subjugate aberrant spindle pole formation by clustering multiple centrosomes to form bipolar spindles. We investigated the contribution of chlamydial infection, in conjunction with HPV E6 and E7 oncoprotein expression, on spindle pole organization. Our findings indicate that a cervical carcinoma cell line with the integrated HPV-16 genome, End1/E6E7, innately cluster centrosomes resulting in a high percentage of bipolar spindles, but upon infection with Chlamydia, clustering is inhibited and disorganization of spindle poles occurs. We believe the synergistic effects between Chlamydia and HPV oncoproteins on centrosome numbers and spindle pole abnormalities identify a possible mechanism for Chlamydia as a cofactor in HPV induced cervical cancer formation.

2923/B641

Novel Insights into Iron Acquisition by Chlamydia.

S. Ouellette, C. Mutchler, S. Belkaya, C. Thompson, R. Carabeo; Centre for Molecular Microbiology and Infection, Imperial College London, London, United Kingdom

Chlamydia is an obligate intracellular pathogen that resides within a vacuole called an inclusion. The mechanism by which this pathogen acquires iron is unclear, given its inability to synthesize siderophores. From a screen for small molecules that inhibited chlamydial growth, an inhibitor (79G5) of the host enzyme acylCoA:cholesterol acyltransferase (ACAT) was identified. A quantitative imaging approach was undertaken to observe any changes in the dynamics of transferrin (Tf) transport in the presence of the inhibitor. 79G5 was found to inhibit the recycling of Tf to the plasma membrane, and manifesting its chlamydiacidal effect into iron starvation of the pathogen. Indeed, a known chlamydia iron-responsive gene (mreB) was upregulated by 8 h post-treatment. Because Tf is known to transit through compartments positive for Rab5, Rab4, and/or Rab11, the effects of 79G5 on this transport pathway were monitored in a pulse-chase experiment at various time points. Fluorescent Tf was recycled to the plasma membrane with 70-80% of the signal disappearing by 45 min, while 60% of the signal was retained in 79G5-treated cells. A quantitative analysis of Tf transport to various compartments revealed that 79G5 treatment did not affect the transit of Tf through the fast recycling pathway (Rab5, Rab4/5), but did have a profound effect on the slow recycling pathway (Rab4, Rab11, and Rab4/11). Specifically, Tf accumulated in Rab4 and Rab11 vesicles, and the appearance of the Tf signal in Rab4/11 double positive vesicles was reduced by 50%, when compared to untreated cells, indicative of the importance of Tf transport to Rab4/11 vesicles in chlamydia iron acquisition. In conclusion, the ACAT enzyme critical for iron transport to the inclusion, inhibiting the formation of the Rab4/11 double positive vesicle. Consequently, transport of iron to the chlamydial inclusion is reduced, thus starving chlamydia of this essential metal. Inhibition of growth is maintained regardless of the presence of a fully functional fast recycling pathway of Tf, illustrating the novel observation of a highly specific and selective nature of the interaction of the chlamydial inclusion with the slow recycling pathway.

2924/B642

An Insecticidal Groel Protein with Chitin Binding Activity from *Xenorhabdus Nematophila*.

M. Joshi[2,1], R. Bhatnagar[2], N. Banerjee[1]; [1]International Centre for Genetic Engineering and Biotechnology, New Delhi, India, [2]School of Biotechnology, Jawaharlal Nehru University, New Delhi, India

Xenorhabdus nematophila secretes insecticidal proteins to kill its larval prey. We have isolated a ~58 kDa GroEL homolog, secreted in the culture medium through outer membrane vesicles. The protein was orally insecticidal to a major crop pest *Helicoverpa armigera* with LC50 ~3.6 µg/g diet. For optimal insecticidal activity all the three domains of the protein- apical, intermediate and equatorial were necessary. The apical domain alone was able to bind to the larval gut membranes and manifest low-level insecticidal activity. At equimolar concentrations, the apical domain contained ~1/3 and apical-intermediate domain, ~1/2 bioactivity of the full-length protein. Interaction of the protein with the larval gut membrane was specifically inhibited by N-acetyl glucosamine and chito-oligosaccharides. Treatment of the larval gut membranes with chitinase abolished protein binding. Based on the 3D structural model, mutational analysis demonstrated that surface exposed residues T347 and S356 in the apical domain were crucial for both, binding to the gut epithelium and insecticidal activity. A double mutant T347A, S356A was 80% less toxic ($P<0.001$) than the wild type protein. The GroEL homolog showed a-chitin binding activity with Kd ~0.64 µM and Bmax ~4.68 µmol/gram chitin. The variation in chitin binding activity of the mutant proteins was in good agreement with membrane binding characteristics and insecticidal activity. The less toxic double mutant XnGroEL showed ~8 fold increase of Kd in chitin binding assay. Our results demonstrate that *X. nematophila* secretes an insecticidal GroEL protein with chitin binding activity.

Cell Biology of the Immune System II (2925 – 2943)

2925/B643

The Role of Nox4-Mediated Reactive Oxygen Species Generation in TLR5 Signaling.

M. Seo, Y. Bae; Division of Life and Pharmaceutical Sciences, Ewha Womans University, Seoul, South Korea

Toll-like receptors (TLRs) play key roles in the innate immune response by recognizing patterns in diverse microbial molecules. Among these receptors, Mammalian Toll-like receptor 5 (TLR5) senses flagellin of several bacterial species and activates the innate immune system. In addition to their role in immune response, generation of reactive oxygen species by NADPH oxidase (Nox) also plays an important role in this signaling. However, a link between TLR activation and Nox homologues is poorly understood. In this study, we demonstrated that TLR5 binds with Nox4 resulting in ROS generation in HAECs in response to flagellin. Moreover, TLR5-dependent ROS generation does not require MyD88 which is key role molecule in TLR. Although flagellin activates MAP kinases (ERK, JNK and p38), JNK phosphorylation requires ROS generation induced by Nox4. Pretreatment of JNK inhibitor, SP600125 and knockdown of Nox4 abolished flagellin-stimulated AP-1 activation and cytokine production suggesting that JNK phosphorylation by Nox4 is essential for downstream physiology. Taken together, flagellin-dependent TLR5 activation requires Nox4 activity in which ROS are essential modulators for JNK activation and AP-1 dependent pro-inflammatory cytokine production.

2926/B644

Mycobacterium Ulcerans Induces Expression of Chemokines in Human Keratinocytes via Toll-Like Receptor 2 and 4.

D. Shin[1,3], H. Lee[1,3], K. Kim[1,3], Z. Lee[4], C. Kim[2,3], E. Jo[1,3]; [1]Department of Microbiology, College of Medicine, Chungnam National University, Daejeon, South Korea, [2]Department of Dermatology, College of Medicine, Chungnam National University, Daejeon, South Korea, [3]Infection Signaling Network Research Center, College of Medicine, Chungnam National University, Daejeon, South Korea, [4]Korea Basic Science Institude, Daejeon, South Korea

Mycobacterium ulcerans (MU), an environmental pathogen causes Buruli ulcer, a severe skin disease. The precise roles of pattern-recognition receptors and innate signaling mechanisms remain largely unknown in keratinocytes during MU infection. Here we demonstrate that keratinocyte toll-like receptor (TLR) 2 and partly TLR4, actively participate in the innate immune responses to MU: reactive oxygen species (ROS) generation, and induction of chemokine and cathelicidin expression. Human keratinocytes constitutively expressed TLR2 and TLR4, but actively induction in time dependent manner, after infection with MU. Exposure of keratinocytes to MU resulted in a rapid ROS generation, which was essential for the expression of cathelicidin mRNA and protein. In addition, treatment of keratinocytes with MU resulted in an increase of CXCL-8 and CCL2 expression. Futher, the siRNA knockdown or inhibition studies showed that TLR2, and partly

TLR4, significantly abrogated the MU-mediated expression of CXCL-8, CCL2, and LL-37. Together, this study demonstrates dynamic innate responses to MU via TLR2 and TLR4 in human epidermal keratinocytes

2927/B645

Role of Apurinic/Apyrimidinic Endonuclease 1 for Toll-Like Receptor 2-Dependent Innate Responses in Keratinocytes.

J. Yuk[1,2], D. Shin[1,2], H. Lee[1,2], K. Kim[1,2], E. Jo[1,2]; [1]Department of Microbiology, College of Medicine, Chungnam National University, Daejeon, South Korea, [2]Infection Signaling Network Research Center, Daejeon, South Korea

The skin participates in innate immune defense through toll-like receptors (TLRs), antimicrobial peptides including cathelicidin and defensin gene families, and through activation of hypoxia-inducible factor-1 (HIF-1α). Apurinic/apyrimidinic endonuclease 1/Redox factor-1 (APE1), a ubiquitous 37-kDa protein, functions as a redox activator of various transcription factors including HIF-1α. Although multiple roles of APE1 have been described so far, little is known about the regulatory role of APE1 in keratinocyte innate immune responses. Here we show that APE1 plays a crucial role for transcriptional activation of HIF-1α in response to TLR2/6 stimulation, thus being a key up-regulator in the keratinocyte innate immune responses. In HaCaT cells and primary keratinocytes, TLR2/6 stimulation induced nuclear translocation of APE1 and HIF-1α in a time-dependent manner. In addition, TLR2/6 stimulation induced activation of keratinocyte innate immune effectors, such as LL-37, and β-defensin 2. Further, TLR2/6-dependent expression of innate immune mediators and HIF-1α target gene vascular endothelial growth factor was markedly inhibited by silencing of APE1 in keratinocytes. Taken together, these findings suggest an essential role for APE1 as a key activator of TLR2-dependent innate immune responses in human keratinocytes.

2928/B646

Induction of Anti-tumor Immunity by the Baculovirus Autographa Californica Multiple Nuclear Polyhedrosis Virus.

H. Takaku; Department of Life and Environmental Sciences and High Technology Research Center, Chiba Institute of Technology, Narashino, Japan

M. Kitajima1,4, T. Abe3, Y.Matsuura3, and H. Takaku1,2 Chiba Institute of Technology, Japan, 3Osaka University, Japan, 4Chiba University, Japan. Abstract.presenting-hiroshi.takaku@it-chiba.ac.jp Background Wild type Autographa californica multiple nuclear polyhedrosis virus (AcMNPV) can infect a variety of mammalian cell types in vitro, but it does not replicate in these cells. AcMNPV induces anti-viral cytokine production after injection in vivo, resulting in the protection of cells from infection with vesicular stomatitis virus and influenza virus. Objective We here show that AcMNPV activates immune cells via TLR9/MyD88 signal pathway. We also described the effects of AcMNPV on the induction of immune responsiveness and tumor growth in mice. Results and Conclusions First, we found that AcNPV has the ability to induce innate immune system activation through a MyD88/TLR9-dependent pathway. Next, we examined the effects of AcMNPV in mice on immune responsiveness in general, and anti-tumor immunity in particular. AcMNPV treatment also increased IFN-γ levels in the serum and induced a marked elevation in IFN-γ production by liver mononuclear cells (MNCs) compared to splenocytes. The hepatic MNCs in these animals were found to be highly cytotoxic to NK-sensitive YAC-1 and B16 melanoma cells, but they had little effect on EL4 cells, which are NK-resistant. Intravenous injection of AcMNPV induced NK cell proliferation in the liver and spleen and enhanced anti-tumor immunity in mice that had B16 liver metastases. Furthermore, such treatment increased the survival of C57BL/6, Jα281-/-, and IFN-γ-/-, mice that had been previously injected with B16 tumor cells. Furthermore, AcMNPV efficiently stimulates NK cell-mediated, anti-tumor immunity. the strong immune response induced by AcNPV makes it a promising candidate for a novel, adjuvant-containing vaccine vehicle against infectious diseases. Abe, T. etal., (2003) J. Immunol. 171:1133-1139. Abe, T. et al., (2005) J. Virol. 79:2847-2858. Kitajima, M. et al., (2006) Biochem. Biophys. Res. Commun. 343:378-384.

2929/B647

Regulation and Mechanics of Fc Receptor-Mediated Phagocytosis.

J. van Zon[1], G. Tzircotis[1], E. Caron[1], M. Howard[2,1]; [1]Centre for Integrative Systems Biology, Imperial College London, London, United Kingdom, [2]Department of Systems Biology, John Innes Centre, Norwich, United Kingdom

Eukaryotic cells engulf micrometer-sized particles by a homeostatic process called phagocytosis. In particular, phagocytosis of bacteria and other pathogens by white blood cells is an essential part of our innate immune response. In immune cells, phagocytosis is initiated in part by the binding of Fc receptors to antibodies on opsonized pathogens. Subsequent Fc receptor clustering and phosphorylation leads to a drastic local reorganisation of the actin cytoskeleton, ultimately leading to particle ingestion. How receptor signalling coordinates the intricate spatial and temporal regulation of phagocytosis is still poorly understood. We present opsonized beads to cells transfected with either wild-type (WT) Fc receptors or mutant Fc receptors, where signalling is completely inhibited. We subsequently track the location of Fc receptors and F-actin during phagocytic cup formation by confocal microscopy.We find that only WT Fc receptors form full phagocytic cups, whereas for mutant Fc receptors phagocytic cups stall at an early stage. Surprisingly, for WT Fc receptors we find that phagocytic cups show two distinct types of behaviours: a phagocytic cup either stalls before forming a half-cup or it proceeds until the bead is fully ingested. We can explain these observations using a simple mathematical model that combines receptor diffusion, actin polymerization and mechanics of the cell cortex. In addition, the model reveals a novel feedback loop that ensures that actin polymerization occurs at just the right level to ensure proper particle engulfment.

2930/B648

Drosophila Lipid Droplets Contribute to Innate Immune Response.

S. Cermelli, S. Gross; Developmental and Cell Biology, University of California Irvine, Irvine, CA

Cytoplasmic lipid droplets (LD) are multifunctional organelles present in most eukaryotic cells and are the major form of energy storage in many cells. We recently proposed that droplets also have an important function in storing and chaperoning proteins. In particular, we showed that lipid droplets in Drosophila embryos store large amounts of histone proteins for later use. Previous reports indicate that Histones and histone-derived fragments have antimicrobial properties, but the functional importance of these observations has been controversial, given the fact that histones are believed to predominantly be in the nucleus. However, since histones localize to droplets, we investigated whether

LD-histone proteins have a conserved role in Drosophila. Using a diffusion disc assay we showed that lipid droplets purified from Drosophila embryos have antimicrobial activity against Escherichia Coli. This antimicrobial activity disappeared when lipid droplets were purified in the presence of alkaline carbonate to minimize electrostatic interactions and to decrease level of histones on the lipid droplets. Further, Gel overlay assays confirmed that the lipid droplets killing activity is due to histone proteins. The results suggest that histones may play a role in the defense against infection, and may serve as components of a primitive innate immune response.

2931/B649

Synthetic RGDS Peptide Improves Lipopolysaccharide-Induced Acute Lung Injury by Inhibiting Integrin Signaled MAP Kinase Pathways.

H. Park, C. Moon, J. R. Han, J. L. Kang; Physiology, Ewha Womans University, School of Medicine, Seoul, South Korea

Objective: We investigated the effects of synthetic Arg-Gly-Asp-Ser (RGDS) peptide on key inflammatory responses to intratracheal (i.t.) lipopolysaccharide (LPS) treatment and on the integrin signaled mitogen-activated protein (MAP) kinase pathway during the development of acute lung injury. Methods: mice weighing 19-21 g. Saline or LPS (1.5 mg/kg) was administered i.t. with or without a single dose of RGDS (1, 2.5, or 5 mg/kg, i.p.), anti-αv or anti-β3 mAb (5 mg/kg, i.p.). Results: RGDS inhibited LPS-induced increases in neutrophil and alveolar macrophage numbers in bronchoalveolar lavage (BAL) fluid, the productions or activity of TNF-α, MIP-2, and matrix metalloproteinase-9, and total protein levels in BAL fluid at 4 or 24 h post-LPS treatment. RGDS inhibited LPS-induced phosphorylations of focal adhesion kinse (FAK) and MAP kinases, including ERK, JNK, and p38 MAP kinase activation in lung tissue. Similarly, anti-αv mAb significantly inhibited these inflammatory responses, but anti-β3 mAb was not effective at the increase in BAL protein at 24 h post-LPS. Conclusion: These results suggest that RGDS with high specificity for αv integrin complex may improve pulmonary inflammation as well as edema during LPS-induced acute lung injury.

2932/B650

The Antifungal Agent Itraconazole Interferes with Glycoprotein Processing in Macrophages.

T. Frey[2], A. De Maio[1,2]; [1]Department of Surgery, University of California San Diego, La Jolla, CA, [2]Graduate Program in Cellular and Molecular Medicine, Johns Hopkins University, Baltimore, MD

Itraconazole (ICZ) belongs to the family of azole antifungals, which are widely used in humans. The antifungal action of this drug is mediated by blocking ergosterol production, which is required for cell growth. In mammals, ICZ inhibits the cholesterol biosynthetic pathway, at the level of the cytochrome P450 enzyme 14-α-demethylase, which catalyzes the eventual conversion of lanosterol into cholesterol. Incubation of macrophages with ICZ alters the processing of glycoproteins from high mannose structures to complex glycans. Thus, all newly synthesized glycoproteins, as detected by the incorporation of [2-3H]-mannose, are endo-H sensitive in cells treated with ICZ. In addition, cells treated with the drug showed increased surface Con A binding, corroborating an increase in high mannose glycoproteins on the plasma membrane. These observations were confirmed by 2D-HPLC analysis of glycans isolated from ICZ-treated cells. The effect of ICZ on glycosylation was not dependent on a reduction of cellular cholesterol levels or inhibition of the cholesterol biosynthetic pathway. Our results suggest that, directly or indirectly, ICZ affects the activity of GlcNAc transferase I or the transport of UPD-GlcNAc into the Golgi. Finally, the effect of ICZ was specific for this drug since other azole antifungals, such as ketoconazole and econazole, did not alter glycan processing. These results demonstrate that ICZ treatment affects glyconjugate processing and may alter cellular function, homeostasis, and the immune system.

2933/B651

Fenvalerate-Induced Oxidative Stress in Serum of Rats and Its Attenuation by Selected Antioxidant Micronutrients.

A. M. Attia, S. G. El-Banna, F. R. Nomeir, S. El-Besrawy; Environmental Studies, Institute of Graduate Studies & Research, Alexandria, Egypt

The aim of this study was to investigate the propensity of fenvalerate (FEN) to induce oxidative stress in male rats and its possible attenuation by omega-3 and selenium (Se). Rats were pretreated orally with omega-3 plus Se (20 mg/kg and 1 mg/kg BW, three times a week for three weeks) prior to oral administration of a single dose of FEN (20 mg/kg BW). FEN increased ($P<0.001$) serum thiobarbituric acid-reactive substance (TBARS) levels, levels, superoxide dismutase (SOD) and catalase (CAT) activities and inhibited acetylcholinesterase (AChE) activity. omega-3 and Se administration increased the activities of SOD, and catalase, decreased TBARS levels and improved AChE activity. Administration of FEN significantly increased ($P<0.01$) total lipid (TL), cholesterol, triglyceride (TG), and low density lipoprotein (LDL), while the high density lipoprotein (HDL) levels decreased. Omega-3 plus Se decreased the levels of lipids and lipoproteins, and normalized the harmful effects of FEN. FEN administration increased ($P<0.01$) serum aminotransferases, phosphatases, lactate dehydrogenase (LDH), gamma glutamyl transferase (γ-GT), urea, creatinine, uric acid and total bilirubin levels and significantly ($P<0.01$) decreased serum totals protein, albumin and globulin levels. The results suggest that omega-3 plus Se may ameliorate FEN-induced biochemical parameters, lipid peroxidation and altering antioxidant defense system in rats

2934/B652

The Effects of Exercise and of Atmospheric Oxygen Concentration in Normobaric Environment on the Immune Response of Peritoneal Macrophage in Mice.

J. Park[1], K. Chun[2], H. Seo[1], H. Park[1], K. Lee[1]; [1]Department of Microbiology, Yonsei University Wonju College of Medicine, Wonju, South Korea, [2]Pediatrics and Adolescent Medicine, Yonsei University Wonju College of Medicine, Wonju, South Korea

The present study investigates the nature in which the exercise and the atmospheric oxygen concentration in normobaric condition have influence over the weight, the levels of nitric oxide (NO) and reactive oxygen species (ROS), both of which are important anti-microbial factors, produced, and the phagocytic functions of the peritoneal macrophage in the experimental subjects. The experimental subjects were male Balb/c mice within 12 hours of birth. They were randomly divided into four groups: normoxia control group (NC, 21% O2), hypoxia control group (HC, 13% O2), normoxia exercise group (NE, 21% O2), and hypoxia exercise group (HE, 13% O2). Each of the exercise

groups was subjected to travel 18 m/min for the duration of 45 min/day, 3 days/week, for 10 to 20 weeks, beginning on the 10th week of experimental conditions. Then, the concentrations of NO and ROS produced were measured, followed by the observation of the levels of phagocytic activities in the macrophages. The normoxia groups weighed more than the hypoxia groups. The NC group weighed significantly more than the other three groups. Nevertheless, the NE group weighed more than either of the hypoxia groups. Higher concentrations of LPS-stimulated NO were found in the hypoxia groups than in the normoxia groups. Furthermore, no distinguishable differences in the NO concentrations between HE and HC, as well as between NE and NC, were observed. However, normoxia groups had more LPS-stimulated ROS present than the hypoxia groups. Additionally, among both normoxia and hypoxia groups, the exercise groups had more LPS-stimulated ROS present than their respective control groups. Finally, the HE group was observed to exhibit the most active phagocytosis, followed by the HC group. The NE group was observed to be more phagocytic than the NC group. In conclusion, the immune responses of peritoneal macrophages in mice are influenced by exercise as well as by the atmospheric oxygen concentration. However, exercise and atmospheric oxygen concentration influence the response through different pathways. To understand more about the actual mechanisms of how each factor influences the immune response, more research and experiments should be conducted.

2935/B653

Inhibitory Effects of a Japanese Traditional Medicine (Kampo Medicine), Shosaikoto, on Inflammatory Responses in LPS-Treated Human Gingival Fibroblasts.

P. Wang, T. Ara, T. Hattori, Y. Imamura; Dept of Pharmacology, Matsumoto Dental University, Shiojiri, Japan

Objective Periodontal disease is known to increase the risk of several diseases such as aspiration pneumonia, infective endocarditis, arteriosclerosis, diabetes and cancer. Although acid non-steroidal anti-inflammatory drugs (NSAIDs) were administrated to improve gingival inflammation, these drugs frequently induce gastrointestinal dysfunction. Shosaikoto has anti-inflammatory effect and is used clinically to treat various inflammatory diseases such as chronic hepatitis. For the purpose of clinical application of Shosaikoto to periodontal disease, we first investigated the anti-inflammatory effects of Shosaikoto *in vitro*. Methods We used *in vitro* periodontal disease model, in which human gingival fibroblasts (HGFs) treated with 10 ng/ml of LPS from *Porphyromonas gingivalis* produce IL-6, IL-8 and prostaglandin E_2 (PGE_2). Shosaikoto (0.1, 0.3 and 1 mg/ml) was added alone or concomitantly with LPS. The amounts of IL-6, IL-8 and PGE_2 were measured by ELISA. Cell viability was evaluated by MTT assay. The expressions of phospholipase A_2 (PLA_2) and cyclooxygenase (COX)-2 were examined by Western blotting. The effect of Shosaikoto on COX activity was measured using 'COX inhibitor screening assay' kit. Results Treatment with LPS, Shosaikoto (up to 1 mg/ml) and their combinations for 24 h did not affect the viability of HGFs. Moreover, Shosaikoto did not alter LPS-induced IL-6 and IL-8 productions. However, Shosaikoto significantly suppressed LPS-induced PGE_2 production in a dose-dependent manner while Shosaikoto alone did not affect basal PGE_2 level. Western blotting demonstrated that Shosaikoto decreased LPS-induced COX-2 expression in a dose-dependent manner but not PLA_2. Moreover, Shosaikoto inhibited selectively COX-2 activity to approximately 50% at 1 mg/ml. Conclusion These results suggest that Shosaikoto decreased PGE_2 production by inhibition of both COX-2 expression and activity and that Shosaikoto may be useful to improve gingival inflammation in periodontal disease. Moreover, because Shosaikoto did not affect basal PGE_2 level, Shosaikoto may have minimal gastrointestinal dysfunction.

2936/B654

Role of Pold3 Accessory Subunit in AID-Mediated Mutagenesis and Recombination.

V. Poltoratsky, J. K. Horton, S. H. Wilson; LSB, NIEHS, Research Triangle Park, NC

Stimulation of mature B cells during acquirement of humoral immunity leads to accumulation of deoxyuracil in DNA encoding the light and heavy chain immunoglobulin. This process is initiated with the conversion of cytosine to uracil by the enzymatic activity of activation-induced cytidine deaminase (AID). In the canonical base excision repair (BER) pathway, these lesions are removed by uracil-DNA glycosylase (UDG) leaving the toxic apurinic/apyrimidinic (AP) site. Following incision of the AP site by AP endonuclease, DNA polymerase (pol) β conducts gap-filling and removal of the 5'-sugar phosphate. However, such pol β-mediated gap-filling is inappropriate for somatic hypermutation (SHM) since it is relatively error-free. Clearly, if BER is involved in SHM, a DNA polymerase other than pol β is recruited to the gapped BER intermediate. Our previous results, as well as studies in mouse models, have indicated that pol β has an insignificant role in SHM. Pol δ is one of the major replicative DNA polymerases in eukaryotic cells. It consists of four subunits of sizes 125 kDa (polD1), 66 kDa (polD2), 48 kDa (polD3) and 12 kDa (polD4). In contrast to the catalytically active polD1 subunit, polD3 is not essential and yeast strains lacking this subunit are viable but sensitive to DNA damaging agents. In the current study, we utilized a previously developed yeast system to test the role of polD3 in uracil-mediated mutagenesis. We found that the frequency of the AID-dependent mutations and recombination events in yeast lacking polD3 is severely diminished compared to the wild-type isogenic strain. In addition, an in vitro assay demonstrated that uracil-mediated BER is also diminished in this mutant strain. Using laser micro irradiation and live cell microscopy, we demonstrated that the polD3 subunit is recruited to sites of DNA damage. Consistent with data obtained in the yeast model, reduction in polD3 expression in Raji lymphocytes resulted in moderate sensitivity to DNA damaging agents and blocking of class switch recombination (CSR). Current studies are addressing the role of pol δ in CSR and SHM.

2937/B655

Recycling Endosome Mediated Secretion of Cytokines in NK Cells.

E. Reefman, J. Kay, A. Manderson, C. Offenhauser, D. Brown, J. Stow; IMB, University of Queensland, St Lucia, Brisbane, QLD, Australia

Natural killer (NK) cells are renown for their ability to kill virally infected or transformed host cells by release of lytic granules containing granzymes and perforin. NK cells also have important regulatory capabilities, which are chiefly mediated by secretion of cytokines such as interferon-gamma (IFN) and tumor necrosis factor alpha (TNF). The secretory pathway utilized for the release of cytokines is unknown in NK cells. Here, we show the localization and trafficking of TNF and IFN in NK cells in compartments and carriers that do not overlap with perforin or other late endosome(LE)-granule markers. Post-Golgi TNF and IFN show significant overlap with markers of recycling endosomes (RE). While perforin granules are released at the synapses in a polarized fashion, TNF and IFN are secreted in a non-polarized fashion all over the cell surface. RE are functionally required for cytokine release since inactivation of RE or mutation of RE related proteins

Rab11 and Vamp3 blocked cytokine surface delivery and release. In contrast, RE are not needed for release of perforin granules, however formation of granules is dependent on functional RE. Thus, RE differentially regulate the release of multiple immunological mediators from NK cells.

2938/B656

In Vitro Differentiation and Functional Characteristics of Human Monocyte-Derived Dendritic Cells — Potential Antigen-Specific Immunomodulators for Cancer Patients.

M. A. Azevedo, S. C. Olenscky Gilli, F. F. Costa, S. O. Saad; Hematology and Hemotherapy Center, State University of Campinas, Campinas, Brazil

Introduction and objectives. Dendritic cells (DC) are specialized antigen presenting cells that have a potent capacity of activating immune responses. Many studies have been performed to verify the efficacy of the DC vaccine in cancer patients; not always, however with satisfactory results. To obtain potentially applicable DCs for vaccination, we investigated the phenotype and functional characteristics of in vitro monocyte derived DC using several cytokines cocktails, as well as some of the methods used for tumor associated antigen loading. Methods and Results. The monocytes isolated from peripheral blood of healthy volunteers using a hyperosmotic Percoll density gradient, were cultured for 7 days in RPMI with 10% FCS and different combinations and concentrations of GMCSF, IL4, IFNα (10-400ng/ml), IFNγ and TNFα (30ng/ml). DC differentiated with IFNα+GMCSF (50ng/ml; IFNαDC) or IL4 + GMCSF+IFNγ (30ng/ml, IL4DC), showed stabilized matured phenotypes (up regulation of CD83, CD80, CD86, HLADR and CD40; up to 80% of purity). Functional differences were demonstrated between these DC types, with a higher stimulatory capacity of IL4DC for induction of autologous WT1 antigen specific T lymphocytes. In addition, IL4DC showed higher activation of NFκB pathways, probably involved with the observed higher IL12 production, suggesting a preferential activation of TH1 immune responses. We demonstrated that mRNA-electroporation is an efficient strategy for antigen-loading DC, with good cellular viability (up to 80%), efficacy (40% overexpression of WT1 RNA) and a higher, or at least similar, immunostimulatory capacity than peptide pulsed DC. The mRNA was transcribed in vitro from cloned WT1 gene. IL4DC also showed higher migratory capacity (measured in transwell plates) than IFNαDC, migrating preferentially to CCL3. When submitted to in vitro stimuli with CD40L (20ng/ml, 12hrs), the migration were to CCL21, suggesting that, in vivo, these cells migrated to lymphoid organs after a previous contact with lymphocytes in the inflammation site. Conclusion. These results represent a basis for optimal in vitro DC generation, which could be clinically used to modulate immune responses in cancer. Financial support: CAPES, FAPESP.

2939/B657

Novel Function for VEGF as a Stimulator of Macrophage Apoptosis during Resolution of Inflammation.

M. Petreaca, M. Martins-Green; Cell Biology and Neuroscience, University of California Riverside, Riverside, CA

Wound healing involves a series of sequential phases: inflammation, granulation tissue formation, and remodeling. Initiation and resolution of each phase is precisely regulated, so that their dysregulation impairs the healing process; indeed, many chronic wounds exhibit a prolonged inflammatory phase, suggesting altered resolution of wound inflammation. Resolution of inflammation involves apoptosis of inflammatory cells. However, the mechanisms regulating their apoptosis are incompletely characterized. We have found that inhibition of Vascular Endothelial Growth Factor (VEGF) in rabbit wounds led to increased macrophage numbers and presence in wounds, suggesting VEGF involvement in their removal from the wound. We hypothesized that VEGF stimulates apoptosis of wound macrophages. Therefore, we investigated the role of this growth factor in macrophage apoptosis and have identified mechanisms involved in this process. In cultured human macrophages, VEGF decreased macrophage survival, increased Annexin V-FITC staining and caspase activation. These effects are mediated by VEGFR1 but not VEGFR2, and occur through increased expression of a pro-apoptotic factor, Tumor Necrosis Factor Superfamily Member 14 (TNFSF14/LIGHT). We used microarray, RT-PCR, and immunoblot analyses to show that VEGF increases expression of LIGHT in macrophages. LIGHT stimulates macrophage apoptosis, and does so through the Lymphotoxin β Receptor (LTβR); soluble LTβR, LTβR-Fc, inhibits LIGHT-induced apoptosis, whereas an LTβR agonist alone induced macrophage apoptosis. Furthermore, LIGHT expression after VEGF treatment mediates VEGF-induced macrophage apoptosis; incubation with LTβR-Fc or a LIGHT neutralizing antibody abolished VEGF-induced apoptosis. These results identify a novel role for VEGF through LIGHT in macrophage apoptosis during resolution of inflammation. These findings also have implications for tumor biology; VEGF may play a dual role in tumors, inducing angiogenesis while reducing inflammation, both of which are permissive for tumor growth. Given that VEGF inhibition is being used to treat human cancers, complete elucidation of its functions is important in deciding how to better use VEGF inhibition in anti-tumor therapy.

2940/B658

Interaction between Antibody-Diversification Enzyme AID and Spliceosome-Associated Factor CTNNBL1.

K. Ganesh[1], S. Conticello[1,2], K. Xue[1], C. Rada[1], M. Neuberger[1]; [1]Protein and Nucleic Acid Chemistry, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom, [2]Core Research Laboratory, Instituto Toscana Tumori, Florence, Italy

Activation Induced Cytidine Deaminase (AID) generates the diverse antibody repertoire required to mount a successful immune response. AID deaminates deoxycytosine residues in the immunoglobulin (Ig) locus to deoxyuracil, triggering DNA repair mechanisms that lead to somatic hypermutation and consequently the affinity maturation of antibodies. AID also attacks the immunoglobulin switch region, triggering the IgM to IgG/IgA/IgE class-switch. Mistargeting of AID's mutator activity is dangerous and indeed underpins many of the mutations and translocations in B cell malignancies. The mechanism which normally ensures the proper targeting of AID is, however, unknown. In this study, by use of two-hybrid and co-immunoprecipitation assays we identify CTNNBL1 as an AID-specific interactor. Mutants of AID that interfere with CTNNBL1 interaction yield severely diminished hypermutation and class switching, whilst retaining in vitro deamination activity. Targeted inactivation of CTNNBL1 in DT40 B cells also considerably diminishes IgV diversification. CTNNBL1 is a previously uncharacterised nuclear protein containing armadillo repeats. We show that CTNNBL1 associates with the Prp19 complex of the spliceosome, interacting with its CDC5L-component. The results therefore identify residues in AID involved in its in vivo targeting and suggest they might act through interaction with CTNNBL1, giving insight into the linkage between AID recruitment and co-transcriptional splicing of target genes.

2941/B659

CD98hc Enables B Cell Clonal Expansion and Adaptive Humoral Immunity.

J. M. Cantor[1], C. Browne[2], R. C. Rickert[2], M. Ginsberg[1]; [1]Medicine, University of California San Diego, La Jolla, CA, [2]Program of Inflammatory Disease Research, Burnham Institute for Medical Research, La Jolla, CA

Rapid proliferation of antigen-specific lymphocytes is required for adaptive immunity. CD98hc is a vertebrate membrane protein originally described as a T cell activation marker, but its function has remained unclear in lymphocytes. CD98hc has been studied largely for its role in cellular transformation and anchorage-independent growth. In order to define the role of CD98hc in humoral adaptive immunity, we used conditional gene targeting in mice to delete CD98hc in B cells. We found that CD98hc is absolutely required for B cell clonal expansion. Surprisingly, deletion of B cell CD98hc did not affect B cell maturation, subsets, or morphology of secondary lymphoid organs, but did result in reduced antibody responses to T-dependent or -independent antigens. The mechanism of this effect did not involve interference with early signaling from antigen receptors, but instead was due to total suppression of rapid B cell proliferation, and consequent inability to form plasma cells. CD98hc can mediate both integrin signaling and amino acid transport. Reconstitution with CD98hc mutants revealed that its integrin interaction is required and its amino acid transport function is dispensable for B cell proliferation. CD98hc orthologues and CD98hc-binding integrins appear in primitive vertebrates co-incident with adaptive immunity. These data establish that CD98hc enables clonal expansion of B cells, show that this function is performed by support of cell proliferation through interaction with integrins, and suggest that the survival advantage conferred by adaptive immunity drove the emergence of CD98hc and its ability to capacitate integrin signaling.

2942/B660

Knockout of TRB2 Influences the Functions of Hematopoietic System of Mouse.

G. Lin, J. Yen; Institute of Biomedical Sciences, Academia Sinica, Taipei, Taiwan

Human tribbles homolog 2, TRB2, has been proposed to be involved in cytokine withdrawal induced apoptosis in hematopoietic TF-1 cells. TRB2 functions as a pro-apoptotic molecule and promotes Mcl-1 degradation through caspase-dependent pathway. On the other hand, it was suggested that mouse TRB2 might act as an oncogene and induces acute myelogenous leukemia through a mechanism involving inactivation of C/EBP alpha. In order to know the physiological functions of TRB2, we generated the TRB2 deficient mice by conventional gene targeting approach. Disruption of TRB2 gene influences the status of the hematopoietic system. The values of red blood count (RBC) and hemoglobin in peripheral blood were reduced in TRB2 KO mice and these defects were significantly enhanced in response to phenylhydrazine induced hemolytic anemia. In the other hand, the white blood count (WBC) and number of lymphocytes were elevated in peripheral blood of TRB2 KO mice. Besides, we also found a high level of CD11b+Gr-1high neutrophils in bone marrow of TRB2 KO mice. Finally, it was observed that the induction of inducible nitric oxide synthase (iNOS) and the production of nitric oxide by bacteria stimulation is impaired in TRB2 deficient macrophages. Altogether, these data suggest that knockout of TRB2 might influence the functions of hematopoietic system of mouse.

2943/B661

Molecular Basis of Nerve-T Cell Interaction in an In Vitro Coculture Approach.

M. Nakanishi, T. Furuno; School of Pharmacy, Aichi Gakuin University, Nagoya, Japan

Multiple sclerosis is believed to be mediated by nerve-T cell interaction, but the mechanisms that determine where T cells initiate inflammation are unknown. We are studying neuro-immune interaction with confocal laser scanning fluorescence microscopy to understand how nerve cells communicate with immune cells. Here, an *in vitro* coculture approach was used for cultured mouse superior cervical ganglia (SCG) and T cell hybridomas (K79; Th1 cells). When T cells were added to culture dishes where most neurons showed the outgrowth of long neurites in 48 hr after initiation of SCG neurons, T cells moved to neurites and contacted with them within 2 hrs. Addition of scorpion venom, which activates neurites but not T cells, to the nerve-T cell cocultures increased Ca2+ concentration in T cells. This expected neurite activation was invariably followed by activation of T cells in contact with neurites. The inclusion of propranolol, a β-adrenergic receptor antagonist, dose-dependently suppressed the T cell activation after the increase in Ca2+ in neurites, but the treatment with an α-adrenergic receptor antagonist, did not. These indicate that the neurotransmitter noradrenaline released from the activated sympathetic nerve fibers elicits a direct Ca2+ mobilization in T cells in contact with neurites via β-adrenergic receptors. The result suggests that some chemotactic factors from SCG neurites should induce the movements of T cells to the neurites. We hope this method can be used to study neuro-immune interaction in the pathological conditions.

Cancer IV: Therapeutics (2944 – 2970)

2944/B662

Functional DNA Repair Inhibitor Screens Using a Sub-Nuclear UV Laser Micro-Beam Methodology.

F. Jalali[1,3], J. Wasniewski[1], N. Chan[2,3], S. Harding[2,3], K. Tse[2,3], R. Kumareswaran[3], R. Bristow[1,2,3]; [1]Cellular Imaging Core, STTARR Innovation Facility/ Radiation Medicine Program/ Princess Margaret Hospital, Toronto, ON, Canada, [2]Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada, [3]Applied Molecular Oncology, Ontario Cancer Institute/ Princess Margaret Hospital, Toronto, ON, Canada

The mammalian DNA damage response (DDR) serves to maintain genome stability following exposure to exogenous DNA damage and involves downstream effectors of ATM, DNA-PKcs and ATR kinase activity. Several of these proteins have been considered as possible targets of small molecule inhibitors used to protect against normal tissue damage or sensitize cancer cells to DNA double strand break (dsb)-inducing treatments. Additionally, intra-tumoral hypoxia can alter the DDR response based on altered translation and function of DNA repair proteins. The objective of this study was to determine the efficacy of sub-nuclear UV micro-beam irradiation (IR) as a means to (1) determine recruitment and co-localization of DNA-dsb sensing and repair proteins under oxia and hypoxia and (2), screen novel compounds for their ability to inhibit these endpoints. A unique live cell imaging/ irradiation platform that uses a UV laser coupled into a spinning disk confocal

microscope equipped with custom-made variable-O2 live cell incubator has been developed for this purpose. Quantitative co-localization of proteins was carried out using indirect immunofluorescence and 2-D/ 3-D image analysis. Both UV micro-beam and whole cell IR (2Gy30') of normal (GM5757) and AT (GM5823) human fibroblasts confirmed 3-D and ATM-dependent co-localization (> 0.6 Pearson Correlation) in 3-D nuclear volumes for 53BP1, γH2AX, ATM-Ser1981, MRE11, MDC1 and DNA-PKcs in greater than 80% of cells and across the width of the laser track. In contrast, 53BP1-Ser25 and RPA showed intra-track punctate focal staining suggesting differential localization to DNA single strand breaks. PML did not co-localize within γH2AX-marked laser tracks and residual DNA-dsbs showed similar co-localization at 24h. Hypoxia (0.2% O2; 16h) decreased γH2AX signal by 50% and led to increased residual nuclear DNA damage. Using this methodology, we were able to confirm that small molecule inhibition of DNA-PKcs, MRE11, ATM and PARP pathways leads to deficient protein-protein co-localization under oxia and hypoxia. Our data support the use of sub-nuclear UV micro-beam IR as a useful adjunct to small molecule and siRNA screens for DNA repair inhibitors.

2945/B663

Bacillus Calmette-Guerin Cell Wall Cytoskeleton Enhances Colon Cancer Radiosensitivity through Redox Regulation.

J. Kim[1,2], J. Yuk[3,4], D. Shin[3,4], K. Kim[3,4], E. Jo[3,4]; [1]Radiation Oncology, College of Medicine, Chungnam National University, Daejeon, South Korea, [2]Cancer Research Insititute, Chungnam National University, Daejeon, South Korea, [3]Microbiology, College of Medicine, Chungnam National University, Daejeon, South Korea, [4]Infection Signaling Network Research Center, College of Medicine, Chungnam National University, Daejeon, South Korea

Colorectal cancer is one of the most common cancer in the world, and represents 15% of all cancer types. Mycobacterium bovis bacillus Calmette-Guérin cell wall skeleton (BCG-CWS) consisted of mycolic acids, arabinogalactan and peptidoglycan, has been used for cancer immunotherapy in clinical settings. However, the radiosensitizing effects of BCG-CWS have been largely unknown in anti-tumor therapy. In this study we investigated the radiosensitizing effects of BCG-CWS for colon cancer cells, HCT116, through induction of cell death via generation of reactive oxygen species (ROS). Exposure of HCT116 colon cancer cells to BCG-CWS before ionizing irradiation (IR) resulted in an increase in radiosensitivity. In addition, BCG-CWS plus IR up-regulated ROS generation in a toll-like receptor 2-dependent manner. In addition, BCG-CWS plus IR significantly up-regulated activation of c-Jun N-terminal kinase, but not extracellular signal-regulated kinase 1/2, in HCT116 cells. The activation of apoptosis signal-regulating kinase 1 was also increased by pre-treatment of BCG-CWS before IR. Together, our data indicate that BCG-CWS in combination with IR might be useful as a promising therapeutic strategy to enhance radiotherapy effect in colon cancer cells through ROS-dependent cell death.

2946/B664

Cytokines Differentially Produced by Glioblastoma Enhance Migration of Undifferentiated and Neural Differentiated Human Embryonic Stem Cells In Vivo and In Vitro.

J. Ozolek[1], J. E. Esplen[1], A. Niranjan[2], G. Gobbel[2], W. Fellows-Mayle[2], E. Jane[2], P. Sammak[3]; [1]Pathology, Children's Hospital of Pittsburgh, Pittsburgh, PA, [2]Neurological Surgery, University of Pittsburgh, Pittburgh, PA, [3]Obstetrics, Gynecology, and Reproductive Sciences, University of Pittsburgh, Pittburgh, PA

Objectives: Neural stem cells have been proposed as therapeutic agents for intracranial tumors due to their ability to hone to tumor and tissue injury. The aim was to evaluate if undifferentiated and neural differentiated stem cells selectively migrate in vivo and in vitro to a tumor stimulus and identify specific growth factors (GF) and cytokines that promote migration. Methods: Thirty-six athymic nude mice had intracerebral injections of 1x105 cells consisting of U87 glioblastoma cell line, undifferentiated hES (human embryonic stem cells, HSF-6 and H7 lines; UhES), neural differentiated hES (NhES), and/or sham. Animals were sacrificed at 1, 5, and 10 days post-injection, processed and examined under by laser confocal microscopy. By antibody array, several GFs and cytokines were identified in at least two-fold abundance in U87 conditioned media compared to mouse embryonic fibroblast conditioned media (IL-1α, IL-5, IL-6, IL-7, IL-8, MCP-1, MCP-1α, HGF, GM-CSF). In vitro migration studies consisted of an agar bridge assay and a modified boyden chamber assay using NhES from HSF-6 and H7, a line derived from H9 ES (ENStem-A™) and a fetal neural progenitor line (ReNcell VM). Results: UhES and NhES selectively migrated to the tumor, around the edges, and within tumors. More fluorescence was present at injection sites, less in and around the tumor, and the least at sham sites for days 1, 5, and 10 (except day 10 sham with higher fluorescence than injection or tumor sites). Agar bridge assays showed sporadic migration of NhES cells or colony asymmetry towards U87 cells and HGF, and less so with GM-CSF. ENStem-A™ and ReNcell VM demonstrated increased migration in the presence of cytokines compared to untreated controls. ReNcell had the most robust migratory response to IL-5 and MCP-1 while ENStem-A™ had strong response to IL-7, IL-8, and MCP-1. Conclusions: UhES and NhES show selective migration towards and intermingle with tumor in vivo. A group of select GF and cytokines produced by glioblastoma (IL-1a, IL-6, MCP-1]) seem to be involved in the migratory response. In general, neural progenitors derived from hES appear to have a greater migratory response than neural progenitors derived from fetal brain.

2947/B665

A Comparative Evaluation of the Growth Inhibitory Activity of Antineoplastic Agents against Human Breast Cancer Cell Lines HCC70, HCC1500, and MBD-MB-468.

E. Asemota[2], T. Hawthorne[2], C. L. Douglas[2], K. Parker Johnson[1]; [1]College of Pharmacy, Xavier University of Louisiana, New Orleans, LA, [2]Biology, Xavier University of Louisiana, New Orleans, LA

Cancer cell line models that demonstrate disease progression at different stages of the disease provide useful insight on pharmaceutical intervention for drugs targeting stage specific populations of cells. It is well documented that as cancer cells begin to differentiate the expression of certain receptors changes, often leading to what is known as triple negative cancer. Objective: Commercially available anticancer agents will cause an antiproliferative effect in cell lines derived from African American patients. Therefore, our laboratory used three cell lines from African American donors: HCC1500, HCC70, and MDA-MB-468, which have varying sensitivities to estrogens. The use of these three cell lines may indicate the progression of the disease to triple negative status. The cell lines were used to evaluate the pharmacological response of FDA approved chemotherapeutic drugs. Experimental methods: The alamar blue dye exclusion assay method

was used to determine the antiproliferation caused by these commercially available chemotherapeutic agents in concentrations ranging from 1 millimolar to 1 nanomolar. Results: The data indicated that Raloxifene produced significant growth inhibition at the millimolar and 10-4M concentrations with 31% growth at 10-3M and 31% growth at 10-4M for HCC70, and 31% growth at 10-3M and 34% growth at 10-4M for MDA-MB-468. Conclusion: These data provide useful insight on how to target specific receptors based on the stage of the disease so that novel therapeutics can be synthesized to target cells that have progressed to where multi-drug resistance (MDR) is an issue for consideration. Because many African Americans tend to present with later stages of the disease, novel stage specific drugs may help to lessen the disparity between African Americans and Caucasians.

2948/B666

Small Molecule Rac Inhibitors as Anti-Breast Cancer Invasive Compounds.

A. De La Mota-Peynado[1], E. Hernandez[2], C. Vlaar[2], L. Cubano[1], S. Dharmawardhane[1]; [1]Anatomy and Cell Biology, Universidad Central del Caribe School of Medicine, Bayamon, [2]Pharmaceutical Sciences, School of Pharmacy, University of Puerto Rico-Medical Sciences Campus, San Juan, PR

The objective of this project is to design novel therapeutics targeted against breast cancer invasion and metastasis, the most deadly aspects of the disease. Our previous studies have shown that Rac, a key signaling intermediate that regulates actin cytoskeletal changes during cell migration/invasion and cancer metastasis, is associated with high breast cancer metastatic efficiency. Unlike the related small GTPase Ras, Rac is not mutated in invasive breast cancer, but hyper-activated. Since Rac proteins are activated by GDP/GTP exchange, inhibition of the interaction of Rac with its guanine nucleotide exchange factors (GEF) is expected to inhibit Rac activity in metastatic breast cancer. Therefore, we tested the effect of a known Rac/Rac-GEF inhibitor NSC-23766 on Rac activity of a highly metastatic human breast cancer cell line, MDA-MB-435. NSC-23766 was not an effective Rac inhibitor at therapeutically useful concentrations in this cell line that exhibits high endogenous Rac activity. Therefore, we synthesized novel NSC-23766 derivatives and tested their efficacy on breast cancer and mammary epithelial cell viability and inhibition of Rac activity. Several NSC-23766 derivatives were found to inhibit Rac activity with higher efficiency than NSC-23766 with minimal toxic effects on normal mammary cells. These compounds also inhibited Rac-mediated lamellipodia extension and breast cancer cell migration. Therefore, we conclude that NSC-23766 derivatives show promise of further development as small molecule inhibitors of invasive breast cancer progression.

2949/B667

Anticancer Effect of Capsaicin in Cisplatin-Resistant Hepatocellular Carcinoma Cell Lines.

S. Lee[1], J. Kim[1,2], J. Park[2], N. Park[1], I. Han[2]; [1]Ulsan University Hospital Biomedical Research Center, Ulsan, South Korea, [2]Department of Biological Sciences, University of Ulsan, Ulsan, South Korea

Cisplatin has been widely used for the treatment of various types of cancer. However, the development of resistance to cisplatin, which is followed by relapse of cancer, is a major limitation in chemotherapy. Therefore, we attempted to explore a potential target and an alternative therapeutic strategy to overcome the cisplatin-resistance. We have identified cisplatin-resistant and -sensitive cell lines among human hepatocellular carcinoma (HCC) cell lines derived from the tumor tissues of Korean patients with liver cancer. Using the HCC cell lines, we investigated the possible role of mitotic kinase Aurora-A in the acquisition of the resistance to cisplatin in HCC cell lines. We have discovered that Aurora-A may be associated with cisplatin-resistance directly or indirectly. Moreover, we analyzed the anticancer effect of capsaicin, the active ingredient of red pepper, in the cisplatin-resistant HCC and found that the treatment of capsaicin induces cytotoxicity in cisplatin-resistant liver cancer cells. Our data suggest that Aurora-A might be a new therapeutic target for overcoming cisplatin-resistance and capsaicin has a potential to be used as chemotherapeutic agent in combination with cisplatin.

2950/B668

Role of Preventive Medicine in the Protection Against Cisplatin-Induced Side Effects.

A. Amin[1], A. Hamza[1], A. Kambal[3], S. Daoud[2]; [1]Biology, UAE University, Al-Ain, United Arab Emirates, [2]Histopathology, Tawam Hospital, Al-Ain, United Arab Emirates, [3]Hematology, Tawam Hospital, Al-Ain, United Arab Emirates

Cisplatin (CIS) is known to induce damage to testis and spermatogenesis and to cause many reproductive dysfunctions in both experimental animals and human. Objective: This study evaluates the protective effects of Hibiscus sabdariffa (Roselle) and Zingiber officinale (Ginger) against CIS-induced reproductive toxicity in rats. The protective effects of Ginger and Roselle against CIS-induced apoptosis in testicular and epididymal sperm cells will also be investigated. Methods: Ethanol extracts of Roselle or Ginger (1 g/kg/day) were given p.o. to male albino rats for 26 days, which began 21 days before a single cisplatin i.p. injection (10 mg/kg body weight). Results: Extracts of Roselle and Ginger reduced the CIS-induced sperm abnormality and enhanced sperm motility as shown in the table (*: mean±SE, n= 5; b $P<0.05$, c $P<0.01$, compared with the control group. Parameters Control Cisplatin Roselle Ginger Testes wts (g/100 g) 1.17±0.03 1.00±0.05b 1.08±0.05 1.10±0.08 epididymid Wts (g/100 g) 0.45±0.02 0.33±0.04b 0.46±0.03 0.40±0.03 Sperm count (million/g) 295.3±22.9 78.7±18.06c 301.7±33.46 205.7±17.1b Sperm motility (%) 79.00±3.32 22.00±4.46c 71.00±2.63 79.00±3.32 Sperm abnormality (%) 2.20±0.37 29.00±5.68c 2.00±0.45 2.20±0.37 Both extracts restored the control level of malondialdehyde (lipid peroxidation marker) in the cisplatin-treated testis. CIS injection induced decline in the levels of superoxide dismutase, reduced glutathione and catalase were significantly reversed to control levels in groups where CIS was preceded by the administration of either Roselle or Ginger. In Ginger and Roselle using both TUNEL and FACS analyses, the attenuation of CIS-induced damage was associated with less apoptotic cell death both in testes and sperms. CIS-induced alterations in the testis lipid peroxidation were markedly improved by plant extracts. Conclusions: Both Roselle and Ginger treatment increased the activities of testicular antioxidant enzymes and restored sperm motility of CIS-treated rats. The protective effects of tested plants are, therefore, suggested to be mediated by their potent antioxidant and pro-apoptotic activities.

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Simvastatin Induces Proliferation Inhibition and Apoptosis in CML Cells.

B. Oh, T. Kim, H. Min, D. Lee; Seoul National University College of Medicine, Seoul, South Korea

Simvastatin is an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, the rate-liminting step for cholesterol synthesis. Simvastatin is known to show antiproliferative effects and are anticipated as a potential drug in the treatment of malignancies including acute myelogenous leukemia (AML) and multiple myeloma (MM). The aim of this study was to determine the effect of simvastain on DNA synthesis, cell cycle progression, and cell proliferation in chronic myelogenous leukemia (CML) cells. To test this approach, cellular proliferation assay based on the quantification of ATP was done on K562, KCL-22, LAMA-84 cell lines and primary CD34+ cells from CML patients. Apoptosis assay by Annexin V staining and Western blot analysis for protein level change were done on CML cell lines. We found that simvastatin inhibited cell proliferation and induced G1 arrest. The ability of simvastatin induced G1 arrest and decrease cell growth is thought to be mediated partly through their upregulation of cdk inhibitor p27kip1. Simvastatin also caused apotosis in both caspase dependent and independent pathway. Finally, Simvastatin inhibited cell proliferation in primary CD34+ cells from CML patients. These in vitro data indicate that simvastatin may have improved in vivo efficacy against CML.

2952/B670

Differential Interference of Chlopromazine with the Membrane Interactions of Ooncogenic K-RAS and Its Effects on Cell Growth.

S. Eisenberg[1], K. Giehl[2], M. Ehrlich[3], Y. I. Henis[1]; [1]Neurobiology, Tel Aviv University, Tel Aviv, Israel, [2]Institute of Pharmacology and Toxicology and Department of Internal Medicine I, University of Ulm Medical Center, Ulm, Germany, [3]Cell Research and Immunology, Tel Aviv University, Tel Aviv, Israel

Membrane anchorage of Ras proteins is important for their signaling and oncogenic potential. K-Ras4B (K-Ras), the Ras isoform most often mutated in human cancers, is the only Ras isoform where a polybasic motif contributes essential electrostatic interactions with the negatively-charged cytoplasmic leaflet. The objective of this study was to explore the mechanism by which the cationic amphiphilic drug chlorpromazine (CPZ) affects the membrane association of oncogenic K-Ras(G12V) and its signaling outcome (cell proliferation vs. apoptosis). Combining live cell microscopy, FRAP beam-size analysis and cell fractionation studies, we show that CPZ reduces the association of GFP-K-Ras(G12V) with the plasma membrane and increases its exchange between plasma membrane and cytoplasmic pools. These effects appear to depend on electrostatic interactions, since the membrane association of another related protein that has a membrane-interacting polybasic cluster [Rac1(G12V)] was also affected, while that of H-Ras was not. The weakened association with the plasma membrane led to a higher fraction of GFP-K-Ras(G12V) in the cytoplasm and in internal membranes, accompanied by either cell cycle arrest (PANC-1 cells) or apoptosis (Rat-1 fibroblasts), the latter being in correlation with the targeting of K-Ras(G12V) to mitochondria. In accord with these results, CPZ compromised the transformed phenotype of PANC-1 cells, as indicated by inhibition of cell migration and growth in soft agar. We conclude that CPZ interferes with the membrane association of constitutively active K-Ras by disrupting its charge-based interactions with the membrane; this is results in mistargeting of the dislodged K-Ras to the cytoplasm and to intracellular membranes, culminating in cell-specific effects on its signaling outcome.

2953/B671

Natural Polyphenols Antagonize the Antimyeloma Activity of Proteasome Inhibitor PS-341 by Direct Chemical Interaction.

T. Kim[1,4], J. Park[2], B. Oh[1,4], H. Min[3,4], T. Jeong[6], J. Lee[7], S. Park[2], D. Lee[4,5]; [1]Department of Molecular and Clinical Oncology, Seoul National University College of Medicine, Seoul, South Korea, [2]Department of Chemistry, Seoul National University, Seoul, South Korea, [3]Department of Tumor Biology, Seoul National University College of Medicine, Seoul, South Korea, [4]Cancer Research Institute, Seoul National University College of Medicine, Seoul, South Korea, [5]Department of Laboratory Medicine, Seoul National University College of Medicine, Seoul, South Korea, [6]National Research Laboratory of Lipid Metabolism and Atherosclerosis, Korea Research Institute of Bioscience and Biotechnology (KRIBB), Daejeon, South Korea, [7]Department of Internal Medicine, Gachon University Gil Medical Center, Incheon, South Korea

PS-341 (Velcade®, Bortezomib) is the therapeutic proteasome inhibitor with antimyeloma activity and polyphenols are well known compounds with antiproliferative effect against tumors. We attempted cotreatment of myeloma cells with PS-341 and polyphenols, expecting a synergistic effect. However, the anticancer activity of PS-341 was blocked by polyphenols. The structural features of polyphenols correlated strikingly with their antagonistic effect; particularly, the presence or absence of vicinal diol moiety was the key element for effective blockage of the anticancer function of PS-341. We infer that the vicinal diols in the polyphenols interact with the boronic acid of PS-341 and convert the active triangular boronic acid of PS-341 to inactive tetrahedral boronate, which abolishes the antimyeloma activity of PS-341. We confirmed this hypothesis by 11B NMR spectroscopy and an in vitro assay on multiple myeloma (MM) cell lines and primary myeloma cells from patients. Based on this, restriction of the intake of natural polyphenols in foods or vitamin supplements should be considered during PS-341 treatment in MM patients.

2954/B672

Effects of 3,3′-Diindolylmethane (DIM) on Migration and Invasion of DU145 Prostate Cancer Cells.

D. Lim, J. Park; Department of Food Science and Nutrition, Hallym University, Chuncheon, South Korea

DIM is a major acid-condensation product of indol-3-carbinol which is present in various cruciferous vegetables such as cabbage, broccoli, Brussels sprouts, and kale. Our previous studies have shown that DIM inhibited not only proliferation of cancer cells but also lipopolysaccharide (LPS)-stimulated inflammation responses in murine macrophages. In the present study, we examined whether DIM inhibits migration and invasion of DU145 human prostate cancer cells. DU145 cells were treated with various concentrations (0 - 10 μmol/L) of DIM with or without 10 μg/L of epidermal growth factor (EGF). Cell migration and invasion assays were performed by using transwell membrane filters. The activity of matrix metalloproteinases (MMPs) and secretion of MMPs, tissue inhibitor of metalloproteinases (TIMPs), vascular endothelial growth factor (VEGF) and uroplasminogen activator (uPA) were estimated by gelatin zymography and Western blot analysis, respectively. DIM inhibited the basal and EGF-induced cell migration and invasion in dose-dependent manners. DIM down-regulated MMP-9 expressions, decreased secretion of VEGF and uPA, and increased secretion of TIMP-2 in DU145 cells. In addition, DIM decreased the levels of Tyr-705 phosphorylated signal transducer and activator of transcription (STAT)3 in unstimulated DU145 cells. Interleukin (IL)-6 increased the levels of phospho-STAT3 (Tyr-705 and Ser-727) in the nucleus, which was dose-dependently inhibited by

DIM treatment. We demonstrated that DIM decreased the migration and invasion of DU145 cells, which may be mediated via inhibition of MMP-9 activity and secretion of VEGF and uPA. In addition, inhibition of STAT3 phosphorylation may be one of the mechanisms by which DIM regulates the expression of proteins involved in the regulation of DU145 cell migration and invasion.

2955/B673

A Transition State Analogue of 5'-Methylthioadenosine Phosphorylase with Potential for the Treatment of Human Non-Small Cell Lung Carcinoma.

I. Basu[1], J. D. Locker[2], T. J. Belbin[3], C. Guha[4], V. L. Schramm[5]; [1]Biochemistry, Albert Einstein College of Medicine, Bronx, NY, [2]Pathology, Albert Einstein College of Medicine, Bronx, NY, [3]Pathology, Albert Einstein College of Medicine, Bronx, NY, [4]Radiation Oncology, Albert Einstein College of Medicine, Bronx, NY, [5]Biochemistry, Albert Einstein College of Medicine, Bronx, NY

Methylthio-DADMe-ImmucillinA (MT-DADMe-ImmA) is an 86 pM inhibitor of 5'-Methylthioadenosine phosphorylase (MTAP), which functions in the polyamine pathway by maintaining cellular S-adenosylmethionine (SAM). Systemic inhibition of MTAP should result in accumulation of MTA and subsequent depletion of SAM in tumor tissues, thereby affecting both MTAP+/+ and MTAP-/- tumor xenografts. We have previously demonstrated that MTAP+/+ FaDu, head and neck squamous cell carcinoma xenograft is sensitive to MT-DADMe-ImmA. We now examine the effect on A549 xenografts, a human non-small cell lung carcinoma cell line with homozygous deletion of the MTAP gene. Immunodeficient Rag2-γC-/- and nude mice, bearing subcutaneous A549 xenografts, received daily doses of MT-DADMe-ImmA, administered orally (9 mg/kg body weight) or injected intraperitoneally (5 mg/kg body weight), 6 or 18 days after tumor implantation. Tumor growth was measured and animals were sacrificed, 42 days after treatment initiation. Primary tumor and lungs were fixed in formalin and stained with hematoxylin and eosin (H&E). Total RNA from untreated and treated tumors were amplified and hybridized to a microarray containing 28,000 cDNA clones. There was significant tumor growth retardation of A549 xenografts following treatment with MT-DADMe-ImmA ($p = 0.008$). There was a significant increase in lung metastases in untreated mice (lung weight, 1.35 + 0.07 gm; 65-75 nodules), compared to those that received MT-DADMe-ImmA (0.49 + 0.05 gm; 7-13 metastatic nodules, $p<0.0001$). H&E tumor histopathology confirmed that the treatment caused a large area of central tumor necrosis, increased fibrous stroma and decreased vascularization. The gene expression profile of MT-DADMe-ImmA-treated A549 solid tumors revealed 65 up-regulated mRNAs ($\geq$ 3-fold expression) including, C-X-C chemokine ligand PPBP and growth inhibitor RERG and 68 down-regulated mRNAs ($\leq$ 3-fold expression) including, ODC1, SMS of the polyamine pathway and inhibitor of apoptosis (IAP) family proteins, BIRC2, BIRC3 and Survivin. MT-DADMe-ImmA demonstrated significant cytotoxicities on human A549 cells in vivo and thus exhibits potential as an agent in the treatment of lung cancer.

2956/B674

Diazinon Induced Lipid Peroxidation and Biochemical Indicators in Rat Serum and Ameliorating Role of Garlic.

A. M. Attia[1], H. M. Nasr[2]; [1]Environmental Studies, Institute of Graduate Studies & Research, Alexandria, Egypt, [2]Department of Pest Control and Environmental Protection, Faculty of Agriculture, Damnhour Branch, Alexandria University, Alexandria, Egypt

Purpose of this study was to investigate the effect of diazinon on lipid peroxidation, biochemical parameters and enzyme activities in male rats and possible role of garlic. Rats were pretreated orally with garlic (90 mg/kg BW, three times a week for three weeks) prior to oral administration of a single dose of diazinon (100 mg/kg BW). Diazinon increased ($P<0.001$) serum thiobarbituric acid-reactive substance (TBARS) levels and inhibited AChE activity. Garlic administration decreased TBARS levels and improved AChE activity. Administration of diazinon significantly increased ($P<0.01$) total lipid (TL), cholesterol, triglyceride (TG), and low density lipoprotein (LDL), while the high density lipoprotein (HDL) levels decreased. Garlic decreased the levels of lipids and lipoproteins, and normalized the harmful effects of diazinon. Diazinon administration increased ($P<0.01$) serum aminotransferases, phosphatases, lactate dehydrogenase (LDH), urea, creatinine, uric acid and total bilirubin levels and significantly ($P<0.01$) decreased serum totals protein, albumin and globulin levels. The results suggest that garlic may ameliorate diazinon-induced biochemical parameters, lipid peroxidation in rats.

2957/B675

Identification of a Small Molecule Activator of the Autophagic Pathway as a Potential Anti-Tumor Agent.

Y. Law[1], M. Wang[3], S. Cheng[2], M. Ng[2], K. To[2], S. Lam[1], P. Chiu[1], C. Ko[2]; [1]Department of Chemistry, The University of Hong Kong, Hong Kong, China, [2]Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong, Hong Kong, China, [3]School of Biological Science, The University of Hong Kong, Hong Kong, China

Autophagy is a self-digestion process of the degradation of cytoplasmic components inside lysosomes. Originally known for its role in cellular homeostasis by recycling of damaged or superfluous organelles, it is becoming apparent that autophagy also participates in various pathological processes including cancer. In addition, emerging evidence suggests that cancer can be targeted by modulating the autophagic pathway. To identify small molecules that modulate autophagic activity, we have conducted a cell-based screening of traditional Chinese medicinal herbal extracts and identified a natural product (AO1) from the rhizome of *Alisma orientale*. We have shown that AO1 induced the activation of AMP-activated protein kinase, leading to the formation of GFP-LC3 puncta, autophagosomes and autolysosomes respectively in various tumor cell lines. AO1 also exhibited significant cytotoxicity (average IC50=25μM) against tumor cell lines of different origins. AO1-induced cell death is autophagy-dependent, as pharmacological inhibition of autophagy (by 3- methyladenine) abrogated AO1-induced cell death. Furthermore, we showed that AO1-induced autophagy and cell death were blocked by intracellular Ca2+ chelator (BAPTA/AM) and an inhibitor of Ca2+/calmodulin-dependent protein kinase kinases (CaMKK-α/β) (STO-609), suggesting that AO1 induced autophagy via Ca2+ mobilization and activation of CaMKK. Together these data suggest that AO1 can be further exploited as an anti-tumor agent. In addition, AO1 can be used in the elucidation of the mechanism of autophagy.

2958/B676

Studies of Ferrite Based Magnetic Nanoparticle Transport Mechanisms and Magnetocytolysis Effects on a Model Cell Culture.

H. L. Rodriguez-Luccioni, M. Latorre, C. Rinaldi, M. Torres-Lugo; Chemical Engineering, University of Puerto Rico, Mayaguez, PR

Suspended magnetic nanoparticles, such as ferrites, are known to dissipate energy under an oscillating magnetic field. Such energy dissipation could be employed to locally raise the temperature inside a tumor to 41-46°C, promoting cell death. This technique is promising in the development of a novel cancer treatment with potentially fewer side effects, compared to radio and chemotherapy. Cytotoxicity experiments, transport analysis, and magnetocytolysis experiments were performed in Caco-2 cells (human colon adenocarcinoma epithelial cells) using carboxymethyl dextran coated ferrite nanoparticles. Cytotoxicity was examined by exposing the cells to various concentrations of nanoparticles for 24 and 48 hours. These studies indicated that cell viability was not affected in the concentration range 0.08-2.0 mg/mL. Nanoparticle uptake analyses were performed, results indicated that a quantity of 0.5-1.5 pg of magnetite/cell are internalized by the cell. Magnetocytolysis experiments were performed by nanoparticle exposure variation time, time of magnetic field application, and the time resting after exposure. Nanoparticle suspensions with a concentration of 2.0 mg/mL were put in contact with the cells for a period of 24 hours and the system was exposed to a magnetic field of 100% power, and a frequency of 260 kHz for 30 minutes. The viability analysis was performed 0 hour, 4 hour and 24 hour after the magnetic field application. Results indicated a significant decrease in cell viability for a magnetic field exposure of 30 min and a resting time of 24 hours when compared to similar field applications but with shorter resting times. These suggest that the process of cell's death is mainly apoptotic. It was concluded that magnetic -dextran nanoparticles were non toxic when entering in contact with a cell culture monolayer for a period of 48 hours, but in the presence of magnetic field this nanoparticles are capable of decreasing cell viability.

2959/B677

Expression of VACM-1/Cul5 cDNA in Endothelial Cells In Vitro Increases Antiproliferative Effect of Thalidomide.

J. C. Pelton, D. Harper, G. Marquez, M. Hledin, M. Burnatowska-Hledin; Biology, Hope College, Holland, MI

VACM-1 (Vasopressin-Activated Ca2+-Mobilizing receptor) is a cul-5 gene, and when expressed in cells in vitro, it inhibits cellular growth. In vivo VACM-1 expression is specific to endothelial cells. Mutation in VACM-1 sequence creates a dominant negative phenotype which reverses these effects and induces angiogenesis in endothelial cells in vitro. Since angiogenesis is an important part of cancer metastasis, factors that regulate these processes are important. Thalidomide, a teratogenic drug, is known to decrease angiogenesis, but the cellular target for the thalidomide effect is not known. In these studies, we tested the hypothesis that in normal endothelial cells expressing VACM-1 protein but not in cells expressing a dominant negative VACM-1 mutant, thalidomide will induce VACM-1 protein expression, and consequently, decrease cell growth. Our results show that the dose dependent effect of thalidomide (0 to 50 µg/ml) decreases growth significantly in cells expressing VACM-1 but not in cells that express the dominant negative VACM-1 mutant. Western blot analysis of these samples shows that thalidomide treatment increases VACM-1 concentration in endothelial cells. These results suggest that thalidomide may be an important regulator of VACM-1 protein expression. This work was supported by the NCI grant R15CA104014 and the department of Biology.

2960/B678

Thirty-Nanosecond Pulsed Electric Fields Induced Mitochondrial Independent Apoptosis in Liver Cancer In Vitro and In Vivo.

X. Chen; [1]Department of Hepatopancreatobiliary Surgery, The 1st Teaching Hospital of Medical School, Zhejiang University, Hangzhou, China, [2]Old Dominion University, Norfolk, VA

Nanosecond pulsed electric fields (nsPEFs) are ultra-short pulses that induce direct electric field and biological effects that initiate apoptosis. Thirty nanosecond pulses ranging in high voltage electric filed from 60 Kv/cm to 120 Kv/cm was administered both on hepatocellular carcinoma Hep1-6 both in vitro and in vivo. Initial application of nsPEFs in vitro demonstrated nsPEFs induced apoptosis in hepatocellular carcinoma Hep1-6 in an electric-filed and pulse number -dependent manner measured by caspase activation that correlated with decrease in cell viability 24 hours post nsPEFs treatment. Caspase activity was independent of calcium mobilization. Apoptotic markers Annexin V and propidium iodide revealed neither phosphatidylserine externalization nor direct electric field effects on membrane permeability. However characteristic apoptotic morphological features of plasma membrane such as blebbing and apoptotic body disruption were observed. Mitochondrial function was tested to define apoptotic pathway. There was no intracellular release of mitochondrial proteins, cytochrome c, second mitochondria-derived activator of caspase, direct IAP biding protein nor apoptosis inducing factor (AIF). Further examination of caspases with isozymes specific initiators indicated electric field -dependent increase in caspase 8 and caspase 9 as well as executioner caspases 3, 6, 7. In vivo application of nsPEFs on 32 mice exhibits 100% efficacy with a total exposure time of only 180 microseconds. The application of high voltage (60 kV/cm), ultra-short (30 ns) electrical pulses applied to murine hepatocellular carcinoma triggers both necrosis and apoptosis resulting in complete regression within an average of 45 days. Moreover, none of this hepatocellular carcinoma recurred for at least 90 days during which time the animals were euthanized. None of the treated mice was found metastasis evidence. The advantages of this therapy are minimum invasive, extremely short treatment time and the absence of chemotherapy side effects. NsPEFs could be applied as a therapeutic candidate for hepatocellular carcinoma.

2961/B679

Dietary Canola Oil Reduces Susceptibility to Chemically-induced Mammary Carcinogenesis.

C. Park, L. Mabasa, K. Cho, A. Fowler; Animal Sciences, North Dakota State University, Fargo, ND

Increasing evidence indicates that it is not the quantity of lipid but the type of lipid intake that influences breast cancer risk. Canola oil is currently the lowest saturated fat-containing vegetable oil and has a very high proportion of mono- and polyunsaturated fatty acids. We postulated that canola oil, due to its omega-6 to omega-3 fatty acids ratio, may have an anticancer effect against human breast cancer development by modulating the immune response leading to breast cancer reduction. The objective of this study was to determine if dietary canola oil reduces susceptibility to chemically-induced mammary tumorigenesis. Forty-two four-week-old female Sprague-Dawley rats were randomly assigned to either the control diet or diet supplemented with 10.7% canola oil. At 50 days of age, all rats were injected with 50 mg of nitrosomethylurea (NMU) per kg of body weight to induce mammary cancer. Tumor development was observed and tumor size and volume measured. Twelve weeks after NMU administration, spleens from non-tumor bearing rats were collected and then cultured for the determination of immune cell proliferation. The canola oil supplemented diet reduced the tumor incidence by 22% compared to the control

diet. The latency period and the average number of mammary tumors per rat were not affected by canola oil supplementation when compared to the control diet. However, mammary tumor volumes were significantly reduced in the canola oil fed group when compared with the control fed group. Canola oil supplementation increased the spleen cell proliferation in the presence of concanavalin A and lipopolysaccharide, suggesting improved activation of splenic T and B cell populations. These results indicate that canola oil rich in oleic acid and omega-3 fatty acids can suppress cancer cell growth, which may be related to an enhanced immune response. Further studies are needed for a better understanding of the mechanisms by which canola oil affects specific immune cell proliferation and tumor growth.

2962/B680

The Effect of Ginkgo Biloba Leaf Extract and Its Active Constituents on the Proliferation and Gene Expressions in Malignant Melanoma Cells.

Y. Zhao, Y. Yang; Biological Sciences, Emporia State University, Emporia, KS

Objective: This study is to investigate the effects of the standardized preparation of *Ginkgo biloba* leaf extract, its terpenoid constituents bilobalide and ginkgolides B, and isoflavones genistein, on the proliferation and gene expressions in malignant melanoma cells (WM1552C). Methods: Proliferation of malignant melanoma cells with or without treatment was examined by MTS proliferation assay; the apoptosis of melanoma cells was examined by DNA fragmentation and Hoescht 33342 vital staining; genomic gene expressions in melanoma cells were determined by microarray analysis. Results: *Ginkgo biloba* leaf extract significantly inhibited the cell proliferation of melanoma cells at concentrations not lower than 32 µg/ml. It reduces about 50% of cell proliferation at the concentration of 250 µg/ml. No evidence of apoptosis was found. The two main terpenoid constituents of *Ginkgo biloba*, bilobalide and ginkgolides B, only significantly inhibited the proliferation of melanoma cell at a relatively high concentration (1 mM). They increased the cell proliferation within the concentration range of 2-125 µM. Thus, bilobalide and ginkgolides B did not seem to contribute to the anti-proliferative effect of *Ginkgo biloba* on melanoma cells in this study. Isoflavone genistein significantly reduced the cell proliferation of melanoma at the concentration of 64 µM or higher. Interestingly, genistein significantly increased the cell proliferation at the concentrations of 4 and 8 µM, but it reduced the cell proliferation at a lower concentration (1uM). Microarray analysis revealed up- or down-regulation of an array of genes involving cell cycle regulation (including cycline-dependent kinase inhibitor p21), DNA replication and DNA repair. Conclusion: *Ginkgo biloba* leaf extract inhibited the proliferation of malignant melanoma cells in a concentration and time-dependent manner. The proliferation-stimulatory or inhibitory effect of the individual active constituent of *Ginkgo biloba* might be determined by its dosage. The inhibition of proliferation may be attributed to the effect of *Ginkgo biloba* on the expression of cell cycle regulation genes.

2963/B681

Role of Estrogen Receptor Isoform Expression on Inhibition of Breast Cancer Progression by Grape Polyphenols.

L. Castillo-Pichardo[1], S. Dharmawardhane[2,1]; [1]Biochemistry, University of Puerto Rico-Medical Sciences Campus, San Juan, PR, [2]Anatomy and Cell Biology, Universidad Central del Caribe, San Juan, PR

Grape polyphenols act as cancer-preventives due to their antioxidant, antiangiogenic, antiinvasive, and antiestrogenic actions. Increased ER expression is associated with breast cancer onset. However, ERα expression can be lost in malignant breast cancers that still retain ERβ expression and highly metastatic breast cancers may lose both ERα and ERβ. We recently reported that combined grape polyphenols are more effective than individual compounds at inhibition of ERα(-), ERβ(+) MDA-MB-231 breast cancer cell proliferation, cell cycle progression, and primary mammary tumor growth (Schlachterman et al., 2008. J. Translat. Oncol. 1:19-27). The objective of this study was to determine the effect of individual or combined grape polyphenols on breast cancer cell functions relevant for cancer malignancy using breast cancer cell lines expressing various ER isoforms. At physiologically relevant concentrations (0.5 and 5µM), combined resveratrol, quercetin, and catechin (RQC) were more effective at reducing cell proliferation and migration of MDA-MB-231 (ERβ+, ERα-) and MDA-MB-435 (ER-) breast cancer cells than individual compounds. The reduced proliferation of MDA-MB-231 cells by grape polyphenols was due to a block in G2 progression. Interestingly, individual or combined grape polyphenols did not significantly affect proliferation of MDA-MB-231 cells ectopically expressing ERα (ERβ+, ERα+). These results show that combined grape polyphenols are more efficient than individual compounds at inhibiting cell functions that regulate breast cancer progression of ER negative metastatic breast cancer cells. Presence of ERβ alone enhanced the inhibitory action of grape polyphenols. To investigate the molecular mechanisms of grape polyphenol action in ERβ(+)ERα(-)breast cancer cells, antibody microarrays to total and phospho-forms of signaling proteins were performed. Combined grape polyphenols reduced phosphorylation/activation of a number of proteins that regulate the cytoskeleton. We conclude that grape polyphenols can act as chemopreventives and therapeutics in ERα negative metastatic breast cancers.

2964/B682

Fucoidan from *Fucus Vesiculosus* Induces Apoptosis in HCT-15 Human Colon Carcinoma Cells.

J. Hyun, H. Boo, S. Kim, J. Kang, M. Kim, E. Yoo, H. Kang; Department of Pharmacology, School of Medicine, Institute of Medical Sciences, Cheju National University, Jeju, South Korea

OBJECTIVE : The antitumor activity of fucoidan from *Fucus vesiculosus*, was investigated in HCT-15, human colon carcinoma cells. METHODS : Cell proliferation was assessed using the MTT assay. The induction of apoptosis was evaluated by the identification of DNA fragmentation, Hoechst staining and cell cycle analysis. The expressions of Bcl-2, Bax, caspase-9, caspase-3, PARP, ERK, p38 and PI3/AKT were detected by Western blot. RESULTS : After HCT-15 cells were treated with the fucoidan, several apoptosis events like as DNA fragmentation, chromatin condensation and the increase of the population of sub-G1 hypodiploid cells were observed. In the mechanism of apoptosis induction by fucoidan, we found that fucoidan decreased the Bcl-2 expression, whereas the expression of Bax was increased in a time-dependent manner compared to the control. In addition, the active forms of caspase-9 and caspase-3 were increased and the cleavage of poly(ADP-ribose) polymerase (PARP), a vital substrate of effector caspase, was observed. Furthermore, the induction of apoptosis was also accompanied by strong activation of ERK and p38 kinase and an inactivation of phosphatidylinositol 3-kinase (PI3K)/Akt in a time-dependent manner. CONCLUSION : These findings provide evidences demonstrating that fucoidan induces the apoptosis of HCT-15 cells,

and the proapoptotic effect of fucoidan is mediated through the activation of ERK, p38 and the block of the PI3K/Akt signal pathway. These results support that fucoidan might have potential for colon cancer treatment.

2965/B683

Induction of Apoptosis in A549 Human Lung Carcinoma Cells by Fucoidan from *Undaria Pinnatifida.*

H. Boo, J. Hyun, J. Kang, S. Kim, M. Kim, E. Yoo, H. Kang; Department of Pharmacology, School of Medicine, Institute of Medical Sciences, Cheju National University, Jeju, South Korea

OBJECTIVE. We examined the anti-cancer effect of fucoidan, a sulfated polysaccharide from *Undaria pinnatifida*, in A549 human lung carcinoma cells. **METHODS.** The viability of A549 cells was determined by MTT assay and the morphology change was detected by Hoechst 33342 DNA staining. The effect of fucoidan on cell cycle of A549 cells were evaluated by flow cytometric analysis. The expressions of caspases, Bcl-2 and Bax were examined by Western blotting analysis. **RESULTS.** When A549 cells were treated with fucoidan, fucoidan exhibited potent anti-proliferation activity against A549 cells. Also, some typical apoptotic characteristics, such as chromatin condensation and the increase of the population of sub-G1 hypodiploid cells, were observed. In the mechanism of apoptosis induction by fucoidan, we examined the changes of Bcl-2 and Bax protein expression levels, and activation of caspases. The fucoidan reduced Bcl-2 expression, but the expression of Bax was increased in a dose-dependent manner compared to the control. Furthermore, the fucoidan decreased the procaspase-3 expression, but induced the caspase-9 activation. The cleavage of PARP [poly (ADP-ribose) polymerase], a vital substrate of effector caspase, was found. **CONCLUSION.** These results indicated that fucoidan induces the apoptosis of A549 human lung cancer cells through the down-regulation of Bcl-2 and the activation of caspase pathway. **Acknowledgements.** This work was supported by Brain Korea 21 program of the Korea Research Foundation.

2966/B684

Two N-Acetyllactosamine Specific Monocot Lectins with Potent Anti-Proliferative Activity.

K. Sharma, V. Dhuna, S. Kamboj; Molecular Biology and Biochemistry, Guru Nanak Dev University, Amritsar, India

Two monocot lectins from underground tubers of Sauromatum guttatum and Arisaema utile found specific for N-acetyl-D-lactosamine and asialofetuin were purified by affinity chromatography on asialofetuin-linked amino activated silica. Both lectins were found to have a homotetrameric structure. Subunit molecular masses of Sauromatum guttatum lectin (SGL) and Arisaema utile lectin (AUL) are 13.0 and 13.5 kDa while their native molecular masses are 52 and 54 kDa respectively. Lectins were inhibited by asialofetuin, a complex desialylated serum glycoprotein and N-acetyl-D-lactosamine (LacNAc), a disaccharide. Amino acid analysis of lectins revealed high content of acidic amino acid, very low content of methionine and complete absence of cysteine residues. Chemical modification studies of amino acids in both lectins revealed the importance of tryptophan and tyrosine residues in lectin-sugar interactions. When excited at 295 nm, SGL and AUL gave fluorescence emission maxima (λmax) at 341 and 342 nm respectively. In vitro antiproliferative effect of SGL and AUL was studied on 11 different human cancer cell lines viz. MCF-7 (Breast), SK-N-SH (CNS), 502713 (Colon), Colo-205 (Colon), HCT-15 (Colon), HT-29 (Colon), SW-620 (Colon), Hep-2 (Liver), IMR-32 (Neuroblastoma), DU-145 (Prostate) and PC-3 (Prostate) using SRB assay. The concentrations of SGL which produced 50% inhibition (IC50) of cancer cell lines viz. SW-620, IMR-32, PC-3, SK-N-SH, Colo-205, HCT-15 and HT-29 was 62, 85, 74, 76, 83, 78 and 66 µg/ml respectively. In case of AUL, IC50 was produced in SW-620, IMR-32, SK-N-SH, Colo-205 and HCT-15 at 81, 72, 73, 63 and 77 µg/ml respectively. In the present investigation SGL and AUL were specific for LacNAc, which has been reported as one of the important cancer markers in literature. In future we will study the in vivo anti-tumor effect of monocot lectins using appropriate animal models and investigate the molecular mechanism for anti-proliferative effect.

2968/B686

Cytotoxic Effects of 22 Medicinal Plants on the 4T1 Murine Breast Cancer Cell Line.

J. Jacob, A. L. Murray, A. J. Reinhart; Wayland Baptist University, Plainview, TX

Ethanolic extracts of twenty two medicinal plants thought to inhibit the cyclooxygenase-2 (COX-2) pathway were tested for their cytotoxic effects on the 4T1 murine breast cancer cell line. Cytotoxic effects of the plant extracts were measured through cell viability assays using MTS, long term colony forming assays, and short term dose response tests at concentrations between 100µg/ml and 1µg/ml. Of the twenty two medicinal plant extracts tested, six were found to be cytotoxic and attenuate the growth characteristics of the cell line at a concentration of 100µg/ml, namely, *Rosemarinus officinalis* (Rosemary), *Sanguinaria canadensis* (Blood Root), *Zingiber officinalea* (Ginger Root), *Withania somnifera* (Ashwagandha), *Curcuma longa* (Turmeric), and *Pterocarpus santalinus* (Red Sandalwood). These six plants were further tested to determine if they induced apoptosis in 4T1 cells by means of the Caspase - Glo 3/7 Assay. Results of the test suggest that blood root and ginger root stimulate apoptosis in 4T1 cells. In addition, results of the dose response tests proved blood root to have a cytotoxic effect on the cell line between concentrations of 10µg/ml and 1µg/ml.

2969/B687

Cytotoxic Effects of 23 Medicinal Plant Extracts on the Human Prostate Cancer Cell Line, PC3.

A. L. Murray, J. S. Jacob, A. J. Reinhart; Wayland Baptist University, Plainview, TX

According to the American Cancer Society, in 2008, there will be an estimated 186,320 new cases and 28,660 deaths due to prostate cancer in the United States alone. Depending on the stage of growth of this cancer, the cells may or may not be testosterone responsive. PC3 cells have been derived from human androgen insensitive prostate cancer cells and have been widely used to study prostate cancer. Recent studies have suggested that the cyclooxygenase-2 (COX-2) pathway is crucial to the growth of many cancerous cell lines, and is in fact over-expressed in many tumor cells as compared to their nontumorigenic counterparts. We have sought to test the hypothesis that medicinal plants which down regulate the COX-2 pathway will also attenuate the growth characteristics of PC3 cells, and thus identify them as possible treatments for prostate cancer. PC3 cells were first treated with 100 µg/mL of each of 23 ethanolic plant extracts, and cell viability was measured using MTS cell proliferation assays and long term colony forming assays. Extracts of Blood Root (*Sanguinari candensis*) were found to cause a substantial decrease in PC3 cell viability, and further dose response studies were conducted. To determine whether the

mechanism for this decrease in cell viability was due to apoptosis, Caspase-Glo 3/7 assays were conducted on cells treated with 100 µg/mL Blood Root. Results indicated an increase in caspase 3/7 activity, suggesting the decrease in cell viability was due to apoptosis.

2970/B688

Celosia Argentea Extract Inhibits the Formation of a KITENIN-Dishevelled-PKCδ Complex in Colorectal Cancer Cells.

J. Kim[1,3], L. Li[1], S. Choi[2,3], K. Kim[3], B. Shin[1,3]; [1]Microbiology, Chonnam National University, Gwangju, South Korea, [2]Biomedical Sciences, Chonnam National University, Gwangju, South Korea, [3]Medical Research Center for Gene Regulation, Chonnam National University, Gwangju, South Korea

Background: KITENIN is a binding partner of a KAI1 metastasis suppressor protein and functions as a scaffold that recruits both Dishevelled (Dvl) and PKCδ through its membrane spanning C-terminal region. Once formed, the complex, a key player in the non-canonical Wnt signaling pathway, stimulates the ERK/AP-1 signaling and polymerization of actin filament via downstream effectors of PKCδ and Dvl, respectively. It was recently reported that KITENIN expression is significantly higher in colon cancer cells and that KITENIN seems to promote metastasis in mouse tumor models. In this study, we have screened plant extract library for inhibition of KITENIN using a cell culture based system. Methods: Upon treatment of colorectal cancer cells with plant extract library, cell morphology was observed. Cells with abnormal morphology were subjected to migration and invasion assay. Subsequently, plant extracts that elicit decrease in migration and invasion were screened for the reduced binding affinity of Dvl and PKCδ to KITENIN. Results: Of plant extracts screened, Celosia argentea extract significantly reduced invasiveness of several colorectal cancer cell lines, inhibited the ERK/AP-1 signaling, disorganized actin cytoskeleton, disrupted the complex formation of KITENIN, Dvl and PKCδ and decreased expression levels of KITENIN and Dvl. Conclusions: It is very likely that the Celosia argentea extract reduces the complex formation of KITENIN, Dvl and PKCδ, which in turn suppresses the ERK/AP-1 signaling and actin cytoskeleton polymerization, leading to the inhibition of cancer cell invasion.

Neuronal Diseases III (2974 – 2989)

2974/B692

Novel Suppressors of Alpha-Synuclein Toxicity Identified Using Yeast.

J. Liang[1], C. Clark-Dixon[1], S. Wang[1], T. Flower[1], T. Williams-Hart[3], R. Zweig[2], L. Robinson[1], K. Tatchell[1], S. Witt[1]; [1]Biochemistry & Molecular Biology, Louisiana State University Health Sciences Center, Shreveport, LA, [2]Neurology, Louisiana State University Health Sciences Center, Shreveport, LA, [3]Biological Sciences, Louisiana State University, Shreveport, LA

The mechanism by which the Parkinson's disease-related protein alpha-synuclein (α-syn) causes neurodegeneration has not been elucidated. The objective of this study was to determine the genes that protect cells from α-syn (wild-type). We used a novel genetic screen that exploited the super sensitivity of Saccharomyces cerevisiae expressing α-syn to killing by hydrogen peroxide. Forty genes in ubiquitin-dependent protein catabolism, protein biosynthesis, vesicle trafficking, and the response to stress were identified. Five of the forty genes—ENT3, IDP3, JEM1, ARG2, and HSP82—ranked highest in their ability to block α-syn-induced reactive oxygen species (ROS) accumulation, and these five genes were characterized in more detail. The deletion of any of these five genes enhanced the toxicity of α-syn as judged by growth defects compared to wild-type cells expressing α-syn, which indicates that these genes protect cells from α-syn. Strikingly, four of the five genes are specific for α-syn in that they fail to protect cells from the toxicity of the two inherited mutants A30P or A53T. This finding suggests that α-syn causes toxicity to cells via a different pathway than these two inherited mutants. Lastly, overexpression of Ent3p, which is a clathrin adapter protein involved in protein transport between the Golgi and the vacuole, causes α-syn to redistribute from the plasma membrane into cytoplasmic vesicular structures. We speculate that Ent3p mediates the transport of α-syn to the vacuole for proteolytic degradation. A similar clathrin adaptor protein, epsinR, exists in humans.

2975/B693

Identifying Genetic Factors Associated with Aging That Influence A-Synuclein Induced Toxicity.

A. L. Knight, S. Hamamichi, M. W. Zhang, K. A. Caldwell, G. A. Caldwell; Biological Sciences, University of Alabama, Tuscaloosa, AL

While significant progress has been made in identifying genetic and environmental factors of Parkinson's disease (PD), the most fundamental susceptibility factor of PD as well as other neurodegenerative diseases remains aging. Pathological features of PD include α-synuclein (α-syn) inclusions called Lewy bodies and progressive loss of dopamine (DA) neurons. Exploiting the advantages of conducting cellular and genetic analyses in *C. elegans*, we have examined how changes associated with aging affect susceptibility to α-syn induced toxicity including neurodegeneration and misfolding in this nematode with an experimentally tenable lifespan (~2 weeks). In *C. elegans*, reduced function of the *daf-2*/*INSR* (insulin receptor) gene significantly increases the lifespan and resistance against cellular stress, which is reversed by loss-of-function mutation in the downstream component of DAF-2/insulin signaling, *daf-16*/*FKHR* (FOXO), demonstrating that DAF-16 transcribes cytoprotective genes. To analyze the functional link between insulin signaling and α-syn-induced neurodegeneration, we generated *daf-2* and *daf-16* worm strains that overexpress *α-syn* and *GFP* in the DA neurons. Analysis of DA neurons in *daf-2* background revealed striking protection from α-syn induced toxicity at day 7 (chronological aging), while *daf-16* mutation enhanced degeneration. Interestingly, DA neuron analysis at the mean life span (day 40, biological aging) revealed no significant protection in *daf-2* background, suggesting that metabolic changes in *daf-2* mutants represent critical factors for neuroprotection. To identify genetic factors associated with these presumed metabolic changes, we generated a *daf-2* strain that overexpresses *α-syn::GFP* in body wall muscle cells to perform systematic RNAi against genetic targets that are up-regulated in *daf-2* background. We have analyzed ~400 genes, and have so far identified 15 genes that, when knocked down, enhanced α-syn misfolding *In Vivo*. As we discern the functional significance of these positives as putative cytoprotective gene products, this study is poised to further reveal a network of genetic factors that better defines the role of the insulin signaling and its influence on aging and neurodegeneration.

2976/B694

Over-expression of GK-NCOAT in Hippocampus Results in Increased O-GlcNAcylation.

S. Ho[1], K. Wang[2], J. E. Kudlow[2], A. J. Paterson[1,2]; [1]Cell Biology, University of Alabama at Birmingham, Birmingham, AL, [2]Medicine, Division of Endocrinology, Diabetes & Metabolism, University of Alabama at Birmingham, Birmingham, AL

O-GlcNAcase has been reported to be a susceptibility gene for Type 2 Diabetes and late-onset Alzheimer's disease, also called Type 3 Diabetes. Indeed, people inflicted with diabetes usually have O-GlcNAcylation dysregulation in many tissues and it is becoming increasingly apparent that diabetics also have a high risk for developing Alzheimer's disease. Although evidence indicates that O-GlcNAcylation dysregulation is involved in the pathogenesis mechanism of Alzheimer's disease, studies in research in animal is lacking. Previously, we have cloned a dominant negative spliced variant of O-GlcNAcase (GK-NCOAT) from brain tissue of the Goto-Kakisaki (GK) rat, a spontaneously occurring model of non-insulin-dependent (Type 2) diabetes. We have also shown that the over-expression of GK-NCOAT in cells causes apoptosis. In the current study, we made a tetracycline-inducible, GK-NCOAT-over-expressing transgenic mouse strain using the CamKII-alpha promoter to limit over-expression to the forebrain. Doxycycline (DOX) treated CamKIIα-rtTA ×TRE-GK-NCOAT bi-transgenic mice showed higher O-GlcNAcylation in the hippocampus pyramidal cells than those not treated with DOX. This area also was positive for transgene expression. DOX-treated CamKIIα-rtTA or TRE-GK-NCOAT showed no increase in O-GlcNAcylation level compared to littermate controls. The technique of electrophysiological recording of CA3-CA1 synapses within the hippocampus is now well established for looking at long-term potentiation (LTP) and memory function. We would like to use these DOX-treated bi-transgenic mice to demonstrate a causal link between long term memory and Alzheimer's disease and O-GlcNAcylation dysregulation through the over-expression of a naturally occurring O-GlcNAcase dominant negative gene.

2977/B695

Nucleotide-Dependent Conformational Changes in the Valosin-Containing Protein VCP/P97 Modulate Interdomain Susceptibility to Caspase-6 Mediated Cleavage.

D. Halawani[2], S. Tessier[3], M. Latterich[2], A. C. LeBlanc[1,2]; [1]Neurology and Neurosurgery, McGill University, Montreal, QC, Canada, [2]Anatomy and Cell Biology, McGill Universtity, Montreal, QC, Canada, [3]IRCM, Montreal, QC, Canada

Active Caspase-6 (Casp6) is conspicuously associated with all major pathological hallmarks at all stages of Alzheimer disease (AD). The active Casp6 localizes to the neuritic plagues, neuropil threads, and the neurofibrillary tangles of affected neurons. Its activation also is observed early in mild cognitive impairment conditions that often precede AD. These results suggest that Casp6 activity could be an early instigator of AD. To better understand the physiological consequences of Casp6 activation in AD neurons, a proteomics approach was used to identify targets of Casp6 activity in primary human neurons. Interestingly, p97/VCP, a protein involved in ubiquitin-dependent pathways, was identified as a putative target of Casp6 mediated cleavage. Our main objective is to determine if p97 is a direct substrate of Casp6 and may be disrupted in AD neurons. The biological function of p97 is contingent on the assembly of a barrel-shaped hexamer which undergoes drastic conformational changes in response to ATP binding and hydrolysis. p97 primary amino-acid sequence encompasses several highly conserved caspase consensus sites that are recognized and cleaved by Casp6 in vitro. Using mass spectrometry, we identified several caspase cleavage sites contained within the NH2-terminal domain and the D1/D2 AAA+ cassettes (VAPD179, DELD307, ETID438, TWED478, VEED725). N-terminal sites are preferentially cleaved in the absence of nucleotide, whereas cleavage within D2 AAA+ cassette occurs only in the nucleotide occupied state. Furthermore, mutation of residue K524, which abolishes ATP binding to p97, also prevented cleavage within the D2 AAA+ cassette. Casp6 cleavage had no effect on p97 total ATPase activity in vitro. However, cleavage at either the N- or C-terminus of p97 will truncate domains essential for adaptor protein interactions associated with p97 functions. Our data demonstrate that p97/VCP is a direct substrate of active Casp6 and suggest that nucleotide driven conformational changes in the substrate may modulate accessibility of the caspase cleavage site. Therefore, Casp6 mediated cleavage may have detrimental effects on p97 housekeeping functions essential for neuronal integrity.

2978/B696

GMF Modulates Aβ1-42 -Induced Glial Activation and Inflammatory Cytokine Production.

A. Zaheer[1,2], S. A. Zaheer[3], S. K. Sahu[2], R. Thangavel[2], B. Yang[3]; [1]Neurology, VA Medical Center & Uuniversity of Iowa, Iowa City, IA, [2]Neurology, University of Iowa, Iowa City, IA, [3]Obstetric and Gynecology, University of Iowa, Iowa City, IA

Glia maturation factor (GMF), discovered and characterized in our laboratory, is a highly conserved protein localized in central nervous system. Previously we demonstrated that GMF is required in the induced production of proinflammatory cytokines and chemokines in brain cells. We now report that ventricular infusion of human amyloid beta peptide1-42 (Aβ1-42) in mouse brain caused glial activation and large increases in the levels of GMF as well as induction of inflammatory cytokine/chemokine known for launching the neuro inflammatory cascade in Alzheimer's disease (AD). To test the hypothesis that GMF is involved in the pathogenesis of AD, we infused Aβ1-42 in the brain of GMF-deficient (GMF-KO) mice. GMF-deficient mice showed reduced glial activation and significantly suppressed proinflammatory cytokine/chemokine production following Aβ infusion compared to wild type (Wt) mice. The decrease in glial activation in the GMF-KO mice is also associated with significant reduction in Aβ induced loss of pre-synaptic marker, synaptophysin, and post-synaptic density protein-95 (PSD 95). Diminution of these Aβ1-42 effects in primary cultures of GMF-KO astrocyte and microglia were reversed by reconstituted expression of GMF. Taken together, our results indicate a novel mediatory role of GMF in neuro-inflammatory pathway of Aβ and its pro-inflammatory functions.

2979/B697

Spinal Motor Neurons Survive after Abnormal Cellular Translocation of α-Internexin in Dystonia Musculorum Mice.

K. Tseng[1], C. Chien[2]; [1]Chung Shan Medical University, Taichung City, Taiwan, [2]National Taiwan University, Taipei City, Taiwan

It is a critical goal for neuroscience researchers to study of the neuronal degeneration and apoptosis for human neurological disease. In this study, we would like to use the neurological mutant, dystonia musculorum (dt) mouse, for the study neurological disease. We examined the distribution of neuronal IF proteins using different approaches to understand the pathological role of neuronal IFs in the spinal motor neurons

from dt/dt mice. Our results indicated that α-internexin shows abnormal translocation in the nuclei of spinal motor neurons in dt/dt mice. This abnormal translocation of α-internexin in the nuclei of spinal motor neurons was also confirmed by Western blotting and immunoelectron microscopy. We further tested the cell death of spinal motor neurons by TUNEL assay, and no TUNEL-positive cells could be identified from spinal motor neurons in dt/dt mice. From our studyss, we demonstrated that the spinal motor neuron, unlike the DRG neuron, can survive after abnormal translocation of α-internexin in the cell nucleus in dt/dt mice.

2980/B698

LRRK2 Regulates Neurite Outgrowth via Modulation of ERM Phosphorylation and Actin Remodeling.

L. Parisiadou[1], C. Xie[1], L. Wang[1], X. Gu[1], X. Lin[1], H. Shim[1], Z. Li[2], H. Cai[1]; [1]Unit of Transgenesis/ Laboratory of Neurogenetics, National Institute on Aging/ National Institutes of Health, Bethesda, MD, [2]National Institute of Mental Health/National Institutes of Health, Bethesda, MD

Parkinson's disease (PD) is a neurodegenerative disorder characterized by selective loss of dopaminergic neurons. Mutations in Leucine-rich repeat kinase 2 (LRRK2) have been linked to both familial and sporadic forms of PD. Among them, the G2019S substitution at the kinase domain of LRRK2 likely increases its kinase activity. A previous study indicated that LRRK2 phosphorylates moesin at threonine 558 in a biochemical assay. Moesin together with ezrin and radixin (ERM) are linkers of F-actin with the plasma membrane. Because the G2019S mutation enhances the phosphorylation of moesin and induces a reduction in neurite length, ERM proteins are possible mediators of LRRK2 in regulating neuron morphogenesis. To test this hypothesis, we examined the phosphorylation of ERM proteins in cultured hippocampal neurons derived from newborn LRRK2 null, wild-type and G2019S transgenic mice. Here we show a positive correlation between the levels of phosphorylated ERM (pERM) and LRRK2 in developing neurons, suggesting that the alteration of pERM is responsible for the abnormal neurite outgrowth of G2019S neurons. We found that the number of pERM-positive filopodia at the distal tips of dendrites and axons was increased in the G2019S neurons; whereas it was significantly decreased in the LRRK2 null neurons. To directly examine whether the increase of pERM contributes to neurite growth defects of G2019S neurons, we blocked the phosphorylation of ERM proteins by the introduction of exogenous polypeptides containing the ERM phosphorylation sites. The peptides application reversed the outgrowth defects. Because pERM anchors F-actin and more F-actin was observed at the growth cones of G2019S neurons, we examined whether increased F-actin content also accounts for the growth defects. Indeed, the disruption of F-actin accumulation at the growth cones rescued the G2019S-dependent effect. Together, our findings establish a connection between LRRK2 expression, ERM proteins phosphorylation, actin remodeling and neurodevelopment, reflecting the importance of a delicate balance between unphosphorylated and pERM in neuron morphogenesis and emphasizing the functional involvement of LRRK2 protein in actin-based cytoskeleton remodeling.

2981/B699

Protective Effect Against Cell Death by Enhancement of FALS-associated SOD1 Mutants Activity after Transduction of PEP-1-HSP27 Fusion Protein.

Y. Lee, D. Kim, M. Jeong, M. Lee, S. Kim, S. Lee, H. Jeong, H. Kang, M. Shin, S. Jang, K. Lee, J. Park, W. Eum, S. Choi; Biomedical Science and Research Institute for Bioscience and Biotechnology, Hallym University, Chunchon, South Korea

Familial Amyotrophic lateral sclerosis (FALS) is a progressive neurodegenetative disorder induced by mutations of the SOD1 gene. Heat shock protein 27 (HSP27) is known as a stress-inducible protein; however the precise role of HSP27 in ALS protection has not been established yet. A human SOD1 and HSP27 genes were fused with a PEP-1 peptide in a bacterial expression vector to produce a genetic in-frame fusion protein which can be transduced into astrocyte cells. Transduced mutant SOD proteins markedly lower HSP27 expression levels in comparison with wild type SOD. The purified PEP-1-HSP27 fusion proteins can be transduced efficiently into the cells and protect against cell death by enhancement of mutant SOD1 activity. Moreover, transduced PEP-1-HSP27 efficiently prevents protein aggregation produced by oxidative stress. These results suggest that HSP27 could be a candidate agent for the treatment of FALS and transduced HSP27 fusion protein may be explored as a potential therapeutic agent for FALS patients.

2982/B700

Transduced Human Tat-Sensitive to Apoptosis Gene (Tat-SAG) Fusion Protein Protects Against ROS Generation and Ischemic Insult.

M. Jeong, D. Kim, S. Kim, S. Lee, Y. Lee, M. Lee, H. Jeong, H. Kang, M. Shin, S. Jang, K. Lee, J. Park, W. Eum, S. Choi; Biomedical Science and Research Institute for Bioscience and Biotechnology, Hallym universoty, Chuncheon, South Korea

Reactive oxygen species (ROS) contributes to the development of various human diseases. Sensitive to Apoptosis Gene (SAG) exhibits antioxidant activity against a variety of redox reagents. However, its antioxidant properties have not been completely defined. In the present study, we investigated the protective effects of Tat-SAG fusion protein against cell death and ischemic insults. When Tat-SAG fusion protein was added to the culture medium of astrocye cells, it rapidly entered the cells and protected them against oxidative stress-induced cell death. Furthermore, transduced Tat-SAG fusion protein significantly suppresses the hydrogen peroxide-induced ROS generation compared to the cells treated only with hydrogen peroxide. Immunohistochemical analysis revealed that Tat-SAG fusion protein prevented neuronal cell death in the CA1 region of the hippocampus in response to transient forebrain ischemia after injection of this fusion protein intraperitoneally into gerbils. Our results demonstrated that SAG acts as an antioxidant molecule by suppression of ROS generation and prevention of neurotoxicity. Thus, we suggest that Tat-SAG fusion protein provides a strategy for therapeutic delivery in various human diseases related to reactive oxygen species (ROS), including stroke.

2983/B701

A Novel Interaction between SIMPLE and PRISM at the Early Endosome: Implications in Charcot-Marie-Tooth Disease Pathogenesis.

S. M. Lee, L. Li, L. Chin; Department of Pharmacology, Emory University, Atlanta, GA

Charcot-Marie-Tooth disease (CMT) is the most common peripheral neuropathy with a prevalence of 1 in 2,500, but the molecular mechanisms underlying this disease remain unknown. Imaging studies of Schwann cells from human patients and rodent disease models of CMT subtypes revealed accumulation of peripheral myelin proteins in cytoplasmic inclusion bodies. These findings suggest that aberrant protein trafficking maybe involved in CMT pathogenesis. Recently, mutations in SIMPLE, a ubiquitously expressed protein of unknown function, were identified as the genetic defects responsible for a demyelinating form of CMT (CMT1C). Sequence analysis of SIMPLE reveals a RING finger motif, a predictive determinant of E3 ubiquitin-protein ligase activity. However, the biochemical function and subcellular localization of SIMPLE remain poorly understood. To investigate the subcellular distribution of endogenous SIMPLE in mammalian cells, we performed immunofluorescence confocal microscopic analyses using an antibody against a conserved region of SIMPLE. Our results indicate that SIMPLE is localized to the early endosome, but not the late endosome, endoplasmic reticulum, or Golgi apparatus. To identify novel protein interactors as potential substrates of SIMPLE, yeast two-hybrid screening of a rat hippocampal cDNA library was performed using full-length SIMPLE as bait. We identified a specific interaction between SIMPLE and a novel SIMPLE-interacting protein, PRISM (protein interactor of SIMPLE), and the interaction was confirmed using coimmunoprecipitation studies. Our results also showed that CMT-linked mutations in SIMPLE, all of which are located at the RING finger motif, have no effect on its interaction with PRISM. Furthermore, triple immunofluorescence confocal microscopic analyses demonstrated a substantial overlap between SIMPLE and PRISM at the early endosome, indicating at least a subpopulation of SIMPLE colocalizes with PRISM at the early endosome. Our findings support a role for SIMPLE in regulation of endosomal trafficking, possibly by ubiquitinating PRISM, and suggest that dysfunctional endosomal trafficking may contribute to the pathogenesis of Charcot-Marie-Tooth disease.

2984/B702

Molecular Mechanisms of Neuroprotection in Amyotrophic Lateral Sclerosis.

S. Sakowski, A. S. Busta, J. Dowling, E. L. Feldman; Neurology, University of Michigan, Ann Arbor, MI

Amyotrophic lateral sclerosis (ALS) is a relentlessly progressive disease with no effective treatment. While the mechanisms that lead to ALS disease onset and progression remain elusive, one commonality across all forms of ALS is the loss of motor neurons (MNs). The cause of this MN loss in ALS is unknown; however, axonal degeneration is regarded as an early event contributing to MN loss and is detected prior to the onset of clinical symptoms. Multiple factors may play a role in early axonal degeneration in ALS including direct injury of distal axons and loss of neurotrophic support. Insulin-like growth factor (IGF-I) is a potent neurotrophic factor with neuroprotective properties in central and peripheral neurons. In NSC34 cells transfected with G93A-SOD1, a mutation associated with familial ALS, IGF-I prevents neurodegeneration evidenced by decreased caspase-3 cleavage and decreased DNA fragmentation. While data supports the therapeutic efficacy of IGF-I in ALS and human trials are currently underway, little is known regarding the mechanisms of IGF-I neuroprotection and the point(s) at which IGF-I asserts its beneficial effects. We are utilizing zebrafish as a model organism to examine the detailed mechanisms of early axonal defects associated with disease onset and to examine the efficacy and mechanisms of IGF-I neuroprotection against ALS MN degeneration in vivo. To model ALS in zebrafish, G93A-SOD1 is transiently expressed following cDNA or RNA injection. Zebrafish ubiquitously expressing G93A-SOD1 exhibit dose-dependent defects in MN outgrowth and axonal branching. Taken together, zebrafish have the potential to be a valuable ALS model organism to understand early events in disease pathogenesis and provide an in vivo system to examine the mechanisms of IGF-I neuroprotection.

2985/B703

Cranial Motor Neuron and Muscle Defects in Kif21a Knock-In and Knock-Out Mouse Models for CFEOM1.

J. Desai[1,2], C. Miranda[4], L. Jia[1,2], B. Fritzsch[3], E. Engle[1,2]; [1]Harvard Medical School, Boston, MA, [2]Childrens Hosptial Boston, Boston, MA, [3]University of Iowa, Iowa City, IA, [4]Ohio State University, Columbus, OH

The congenital cranial dysinnervation disorders (CCDD) include a series of complex strabismus syndromes that include various forms of CFEOM (congenital fibrosis of extraocular muscles) and Duane syndrome. CFEOM1 is a rare inherited CCDD characterized by non-progressive ophthalmoplegia with both active limitation and passive restriction of globe movement. Our lab previously reported that CFEOM1 results from heterozygous missense mutations in KIF21A, a member of the kinesin family of motor proteins. Here we show Kif21a is first detected during mouse development at E9.5, and is expressed in mitotic and postmitotic neurons. Within the developing and adult CNS, Kif21a is expressed in many neuronal populations, including oculomotor, trochlear and abducens motor neurons. Kif21a is also expressed in extraocular (EOM) and skeletal muscle, and detailed analysis of the relative timing of expression reveals that Kif21a first appears in skeletal and extraocular muscles at E14.5, subsequent to its CNS expression. To further understand the role of KIF21A in development and in the etiology of CFEOM1, our lab generated Kif21a knock-out (Kif21a-/-) and knock-in (R954W) mouse lines. Kif21a homozygous knock-out mice die within 24 hours of birth while heterozygous mice appear normal. Kif21a-/- brains are smaller than wild-type littermates and motor neurons in the oculomotor, trochlear, abducens, and facial nuclei are reduced in number. Kif21a heterozygous and homozygous knock-in mice (Kif21aR954W) recapitulate human CFEOM1. Adult Kif21aR954W mice are viable and fertile and exhibit unilateral or bilateral ptosis and globe retraction with variable penetrance. Histopathology of adult mice reveals reduced numbers of oculomotor and abducens motor neurons and aplasia of selective EOMs. In contrast, the position and histological appearance of these EOMs are normal at P0, despite their reduced innervation as assessed by DiI tracer studies, indicating aplasia of EOMs is secondary to innervation defects. Taken together, these data support a neurogenic etiology for CFEOM1. Future studies utilizing both Kif21aR954W and Kif21a-/- mice will help our understanding of KIF21A function in CFEOM1 disease etiology and in neurodevelopment.

2986/B704

High Mobility Group Box 1 Aggravates Excitotoxicity-Induced Neuronal Cell Death via RAGE-MAPK Signaling Pathway.

C. Lim[1], S. Kim[1], J. Shin[1], J. Jeong[1], Y. Jin[1], I. Kim[1], K. Kim[1], M. Kim[3], P. Han[2], J. Lee[1]; [1]Anatomy, Inha University School of Medicine, Inchon, South Korea, [2]Division of Nano Sciences and Department of Life Science, Ewha Womans University, Seoul, South Korea, [3]Biological Science, Inha University, Inchon, South Korea

High mobility group box 1 (HMGB1) was originally identified as ubiquitously expressed nonhistone DNA-binding protein. Recently, it was found to act as a cytokine-like mediator of delayed endotoxin lethality and of acute lung injury. Previously, we demonstrated that HMGB1 is massively released extracellularly immediately after ischemic insult, and that it subsequently activates microglia and induces neuroinflammation in the postischemic brain. Here, we report that HMGB1 was accumulated in NMDA-treated primary cortical culture media, and the conditioned media collected from these cultures were able to induce neuronal death if treated to fresh primary cortical cultures. However, HMGB1-depleted NMDA-conditioned media produced by preincubation with anti-HMGB1 antibody or with HMGB1 A box, or by HMGB1 siRNA transfection failed to induce neuronal death. In addition, treatment with recombinant HMGB1 also induced neuronal death in the primary cortical cultures. Moreover, a series of experiment employing co-immunoprocipitation between RAGE and HMGB1 and treatment of SB203580 and PD98059, p38 MAPK and ERK inhibitors, respectively, suggested that RAGE-p38 MAPK and/or RAGE-ERK pathway might be a molecular mechanism underlying extracellular HMGB1-mediated aggravation and induction of neuronal cell death. These results together our previous reports regarding microglia activation by extracellular HMGB1 indicate that HMGB1 functions as a novel danger signal that aggravate brain damage in autocrine and paracrine manners.

2987/B705

The Evaluation of Transepithelial BSE Agent Transport by In Vitro Bovine M Cell Model.

K. Miyazawa[1,2], T. Kanaya[2], N. Nishida[3], T. Yamaguchi[1], S. Katamine[3], H. Aso[2]; [1]Surgery/ Neuropathology, Yale Medical School, New Haven, CT, [2]Cellular Biology Laboratory, Graduate School of Agricultural Science, Tohoku University, Sendai, Japan, [3]Molecular Microbiology and Immunology, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan

Bovine Spongifom Encephalopathy (BSE) is a fetal neurodegenerative disease in cattle. At present, BSE is a threat to human health because of the appearance of BSE-linked variant Creutzfeldt-Jakob disease. The BSE agent appears to spread to the cattle population through eating rendered meat and bone meal contaminated with BSE infected brain or spinal cord. Therefore it is important to identify the intestinal cell types that transport the BSE agent. Membranous epithelial cells (M cells) of gut epithelium have a high capacity for transcytosis of various viruses and microorganisms. Recently, we succeeded in the establishment of a bovine intestinal epithelial cell line (BIE) and the development of an *in vitro* bovine M cell model. We investigated whether M cells transport the BSE agent in this model. We induced M cells by coculture with lymphocytes (abbreviated as cocultures) on transwell inserts. We compared the infectivity of basolateral medium of transwell inserts between BIE cells without lymphocytes (abbreviated as monocultures) and cocultures after exposure of 1% mouse adapted BSE brain homogenate to the apical surface of both cultures. We used mouse bioassays as well as *in vitro* assays with mouse neuronal cell line GT1-7 for the evaluation of transported infectivity. We found that only GT1-7 cells exposed to basolateral medium of cocultures but not basolateral medium of monocultures accumulated abnormal prion protein (PrPres), a surrogate marker for infection. In addition, we demonstrated that the basolateral infectivity of cocultures was approximately 180-fold higher than that of monocultures by bioassays. These results show that the transcellular transport ability of BIE cells has a crucial role in the uptake of the BSE agent *in vitro*. Accordingly we suggest that M cells can act as a portal site for entry of orally delivered BSE agent *In Vivo*.

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Calpain/Cdk5-mediated MEF2D Down Regulation Involved in PC12 Cell Death Induced by 6-Hydroxydopamine.

M. Kim[1], S. Kim[1], J. Hyun[1], J. Kang[1], H. Boo[1], J. Kang[2], E. Yoo[1], H. Kang[1]; [1]Department of Pharmacology, School of Medicine, Institute of Medical Sciences, Cheju National University, Jeju, South Korea, [2]Department of Neurology, School of Medicine, Institute of Medical Sciences, Cheju National University, Jeju, South Korea

Objective. The biochemical mechanisms for dopamine neuron loss in Parkinson's disease are not clearly defined. Recent studies reported that calpain/cdk5-mediated pathway play a central required role in the down regulation of myocyte enhancer factor 2 D (MEF2D), a critical transcription factor for neuronal survival, in MPTP model. We therefore investigated whether the down regulation of MEF2D is involved in 6-OHDA model, and how transcriptional activity of MEF2D is down regulated by 6-OHDA. **Methods.** The cell viability was estimated using the MTT assay. The changes in nuclear morphology of apoptotic cells were investigated by Hoechst 33342 staining. The levels of MEF2D, Bcl-2, Bax, actived caspase-3, PARP were detected by Western blot. **Results.** We found that 6-OHDA significantly decreased MEF2D level in a concentration/time-dependent manner in PC12 cells. And we conformed that 6-OHDA decreased the Bcl2 expression, whereas the expression of Bax, actived caspase-3 were increased and the cleavage of PARP was observed in a time-dependent manner. Furthmore, we observed that the decrease MEF2D by 6-OHDA in PC12 cells was inhibited by calpain inhibitor PD151746 and cdk5 inhibitor Roscovitine, respectively. **Conclusion.** These results suggest that 6-OHDA-induced the degradation of MEF2D is dependent on both calpain and cdk5 activity, and MEF2D down regulation plays a role in PC12 cell death by 6-OHDA. Acknowledgements This work was supported by Brain Korea 21 program of the Korea Research Foundation.

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Novel Antibiotics, CS682, Attenuates Lipopolysaccharide-Induced Neuroinflammation in BV-2 Microglial Cells.

J. Park, E. Park, H. Chun; Dept. Biotechnology, Chosun Univ., Gwang Ju, South Korea

Inflammation plays an important role in the pathogenesis of Parkinson's disease (PD). Microglia, the resident immune cells in the central nervous system (CNS), act as a pivotal cells in the inflammatory reaction in CNS. Activated microglia can induce expression of inducible nitric-oxide synthase (iNOS) and release significant amounts of nitric oxide (NO) and TNF-α. The antibiotics, CS682, is isolated from Streptomyces with diverse biological effects including anti-inflammation. Activated BV-2 cells induced release of nitric oxide (NO) and proinflammatory cytokines. However, pretreatment with CS682 inhibited microglial activation and reduced the production of proinflammatory cytokines such as TNF-α, IL-1β and IL-6. Thus, CS682 may possess therapeutic potential against inflammation-related neurodegenerative diseases. [Supported by BK21 program of MOE and the SRC/ERC program of MOST/KOSEF(R11-2000-083-02006-0), Korea]